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To the Graduate Council:

I am submitting herewith a thesis written by Paul Richard Ortstadt entitled "Wing bud mesodermal cells that survive in vivo die in vitro by apoptosis." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Zoology.

Jeff MaCabe, Major Professor

We have read this thesis and recommend its acceptance:

Shivers, Kennedy

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a thesis written by Paul Richard Ortstadt entitled "WING BUD MESODERMAL CELLS THAT SURVIVE *IN VIVO* DIE *IN VITRO* BY APOPTOSIS." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Zoology.

Jeff MacCabe, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of Graduate School

WING BUD MESODERMAL CELLS THAT SURVIVE *IN VIVO* DIE *IN VITRO* BY APOPTOSIS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Paul Richard Ortstadt

December 1996

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ABSTRACT

During embryonic development of the chick, several regions of programmed cell death (PCD) occur in the wing bud including the anterior necrotic zone (ANZ), the opaque patch (OP), the posterior necrotic zone (PNZ) and the interdigital necrotic zones (INZ). These regions also undergo cell death in organ culture and are rescued by both basic fibroblast growth factor (FGF-2; a growth factor known to be present in the developing wing bud) and cycloheximide (CHX; an inhibitor of protein synthesis). In this study we examined *in vitro* cell death in tissues from areas of the chick wing bud not normally programmed to die during development.

Regions of stage 21 chick limb bud were excised and placed in organ culture. These regions were comprised of tissue which contained the opaque patch or the posterior necrotic zone or tissue just anterior or posterior to the opaque patch. This study focused on the anterior and posterior tissues and occasionally included the other regions for comparative purposes. The ^{51}Cr release assay was utilized to quantify the amount of cell death occurring in these tissues cultured in media with or without various supplements. Tissues from anterior and posterior to the opaque patch were inspected by transmission electron microscopy for cellular morphology and debris after culture in base medium or in medium supplemented with 200ng/ml FGF-2

or 10ug/ml CHX. DNA from these two regions of tissue was analyzed by gel electrophoresis to assess whether fragmentation of DNA had occurred during the culture conditions employed for the preceding microscopy.

The results of this study illustrate that anterior and posterior regions of tissue which elude the PCD process in the developing chick wing bud are susceptible to apoptotic fate in organ culture. Production of oligonucleosomal-sized fragments of DNA in organ culture indicates that this death is apoptosis. Inhibition of cellular death and DNA fragmentation by an inhibitor of protein synthesis (CHX) further supports this conclusion. The occurrence of apoptosis rather than necrosis in these tissues in organ culture suggests that developmental cell death occurring in some regions of the chick limb bud mesenchyme may result from lack of access to a certain factor or factors. In addition, evidence from the ^{51}Cr release assay and transmission electron micrographs indicates that the apoptosis observed in this study is inhibited by FGF-2. FGF-2 is known to be expressed in the chick limb bud. Cell proliferation and inhibition of apoptosis in these tissues *in vivo* may, in part, be attributable to FGF-2 present in the developing limb bud.

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1. INTRODUCTION

Characteristics of Apoptosis

Cells undergoing programmed cell death (PCD),* referred to as apoptosis, exhibit a number of morphological features that allow them to be distinguished from cells dying by necrosis (Wyllie *et al.*, 1980). Unlike necrotic cells, apoptotic cells are found scattered throughout a tissue rather than in a cluster or clusters of mass death. Chromatin of the apoptotic cell condenses and collects along the nuclear envelope sometimes forming a dark cap. The nuclear membrane loses its pores along condensed chromatin and buckles. Cytoplasm of the cell condenses, and, in compact tissues, the cell pulls away from neighboring cells. Eventually, portions of the cell pinch off to produce apoptotic bodies. Cellular organelles tend to remain intact and may sometimes be packaged with nuclear chromatin in apoptotic bodies. These bodies are often rapidly phagocytosed by adjacent cells within the tissue.

Apoptosis is also characterized by cleavage of DNA into oligonucleosomal-sized fragments by the action of an endogenous endonuclease (Wyllie, 1980). This endonuclease

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* For a complete listing of abbreviations and symbols used in this study, see Appendix B.

may be DNase I and seems to acquire its specificity in cleavage from a certain nuclear factor or factors (Peitsch *et al.*, 1993). Oberhammer *et al.* (1993) have shown that epithelial cells induced to undergo PCD by TGF-*B*1 produce large nucleotide fragments of 300 kilobase pairs (kbp) and 50 kbp prior to or in lieu of production of oligonucleosomal-sized fragments of approximately 180 base pairs (bp). They suggest that this might be facilitated either by chromatin structure or by initially low levels of endonuclease activity. However, Pandey *et al.* (1994) have provided evidence that two separate endonucleases (or stores of endonuclease activity) are employed during apoptosis. They determined that one functions independently of calcium (to produce larger fragments) and one is dependent on the presence of magnesium and calcium (to produce oligonucleosomal-sized fragments).

Nuclear events (if not DNA cleavage itself) may be involved in initial commitment to the apoptotic pathway. Garcia-Martinez *et al.* (1993) noted a loss of potential to form chondrogenic condensations by interdigital chick leg mesenchyme *in vitro* a day and a half prior to visible manifestations of cellular death. This illustrates that developmental decisions can occur within cells long before their ulimate fates are evident. Similarly, Pollak and Fallon (1976) noted that, in the posterior necrotic zone (PNZ) of the chick, DNA (as well as RNA) synthesis decreases

at a time coincident with commitment to the death program 16 hours before morphology indicates the occurrence of cell death. In addition, Toné *et al.* (1988) have shown that before morphological changes associated with apoptosis are evident in interdigital regions (interdigital necrotic zones; INZ) of the chick embryo, cell populations exhibit changes in DNA synthesis and content that are not associated with any portion of the normal cell cycle. Moreover, Toné *et al.* (1994) have shown that nuclei that have not yet become pycnotic may exhibit DNA strand breakage.

Evidence that expression of certain genes within prospective dying cells influences the cell death process comes from discovery of single gene mutations that induce or inhibit PCD (see Schwartz and Osborne, 1994; Steller, 1995; Vaux, 1993; for reviews). In addition, RNA and protein synthesis inhibitors can be used to inhibit cell death (Wyllie *et al.*, 1984; Fahrbach *et al.*, 1994; for example). Synthesis of critical cell death proteins would presumably occur prior to a decrease in protein synthesis, as noted by Pollak and Fallon (1974), in a region of programmed cell death in the chick wing bud.

Developmental Cell Death in the Limb Bud

Cell death evident during development in vertebrates occurs in a predictable manner and may be considered as one

of a number of possible cell fates during development of an organism (Glucksmann, 1951; Saunders and Fallon, 1967). During certain stages of embryonic development of the chick wing bud, regions of programmed cell death are apparent and are involved in formation of the vertebrate limb (reviewed by Hurle *et al.*, 1995). These include the anterior necrotic zone (ANZ) which first develops at stage 24 (reviewed in Hinchliffe, 1982) , the opaque patch (OP) which appears at stage 23 (Dawd and Hinchliffe, 1971), and the PNZ which is prominent at stage 24 (Saunders and Gasseling, 1962; Saunders and Fallon, 1967). The ANZ appears to play an important role in morphogenesis of the avian limb since early advent of the ANZ has been implicated in production of the *wingless* morphotype (Hinchliffe and Ede, 1973). The OP reaches its greatest extent at stage 24 and morphology of the dying cells therein corresponds to that of apoptosis (Dawd and Hinchliffe, 1971; Wyllie *et al.*, 1980). The OP has been suggested as the cause for bifurcation of the distal end of the limb bud's chondrogenic condensations leading to dividing of radius and ulna in the chick wing bud (Dawd and Hinchliffe, 1971). The PNZ, however, appears to be of little importance in determining the shape of the limb (Hinchliffe, 1982).

Cell death also plays a role in avian digit formation, and the amount of cell death observed roughly correlates with the degree or absence of webbing between the toes of

birds (Hurle and Climent, 1987; Saunders and Fallon, 1967). The form of cell death detected in interdigital regions of the chick exhibits characteristics of apoptosis (Garcia-Martinez *et al.*, 1993; Tone *et al.*, 1994). Although cell death is also detectable in interdigital regions of reptiles (Fallon and Cameron, 1977) it does not seem to be utilized in all vertebrates since it has not been found to be involved in formation of the unwebbed digits of amphibians (Cameron and Fallon, 1977).

FGFs and Limb Bud Development

There is abundant evidence that the fibroblast growth factor (FGF) family may play a role in outgrowth of the limb bud. Seed *et al.* (1988) have shown that FGFs are present throughout stage 18 chick embryos and are in higher concentration in the developing limb bud until stage 27 at which point FGF level drops below that detected in the body. In addition, Cohn *et al.* (1995) have shown that soaking beads in either FGF-1, FGF-2, or FGF-4 and placing them in the sides of chick embryos induces limb bud outgrowth. Limb buds expressed *sonic hedgehog* and a homeobox containing gene (which are implicated in antero-posterior polarization of the limb bud) and developed into additional limbs of a type determined by the region of the flank whereat limb bud formation was induced.

FGF-4 transcripts have been detected throughout the apical ectodermal ridge (AER) of the mouse limb bud though in higher concentration along the posterior (Suzuki *et al.*, 1992). Treatment of the mouse limb bud with antisense oligonucleotides for the FGF-4 gene by Ochiya *et al.* (1995) hampered limb development which suggests that expression of FGF-4 is necessary for the proliferation of mesenchyme that results in limb bud outgrowth. Application of FGF-4 and a polarizer (retinoic acid) to the anterior of the limb bud leads to production of homeobox containing gene RNAs in a pattern that is a mirror image of that seen in the untreated limb bud; however, FGF-4 alone will neither induce formation of a polarizing center in the anterior of the limb bud (Niswander *et al.*, 1994) nor stimulate digit formation (Niswander *et al.*, 1993). Although FGF-4 does not seem to be the factor that initiates limb bud outgrowth, it appears that FGF-4 still plays an important role in limb development. *Sonic hedgehog* protein has been discovered to induce expression FGF-4 in the AER, and the two can form a positive feedback loop in which each maintains the other's expression (Niswander *et al.*, 1994). This may account for the link between the zone of polarizing activity (ZPA) and FGFs noted by Aono and Ide (1988).

FGF-8 has also been implicated in limb bud outgrowth. FGF-8 is expressed in limb bud ectoderm of the mouse prior to appearance of the AER, during the AER's development and

in reduced amount as the AER diminishes which suggests that it plays a role in induction of limb bud outgrowth (Ohuchi *et al.*, 1994; Crossley and Martin, 1995). In further support of this, FGF-8, unlike FGF-4 (Suzuki *et al.*, 1992), is not diminished toward the anterior portion of the limb bud's AER (Ohuchi *et al.*, 1994). It is believed that, through alternative splicing, the FGF-8 gene may encode a family of factors (Crossley and Martin, 1995).

Yet another member of the family that appears to be of great importance to development and organization of the vertebrate body plan is FGF-2. Borja *et al.* (1993) have shown that there are at least eight different FGF-2 transcripts, one class of which is expressed more in embryos than in adults and includes a variant whose product binds heparin very strongly. It has been shown that an increase in intracellular levels of FGF-2 can modify cellular motility (Taylor *et al.*, 1993; Tsuboi *et al.*, 1990). In addition, FGF-2 delays death of cell cultures, serves as a mitogen, induces secretion of proteins and maintains the phenotype of cells in culture (reviewed in Gospodarowicz, 1983). It is also required for differentiation of some cell types, delays differentiation (Seed and Hauschka, 1988) and "rescues" cells from death (MacCabe, 1993; MacCabe *et al.*, 1991).

FGF-2 also appears to be of great importance during development of the chick limb bud. Munaim *et al.* (1988) have shown that timing of maximal expression of FGF-2 during

development of the limb bud correlates with the early proliferation of mesoderm therein. Evidence that suggests the AER may be a source of FGF-2 comes from Anderson *et al.* (1994) who note that both the AER and FGF-2 inhibit differentiation of limb bud mesenchyme into ZPA cells. In addition, Fallon *et al.* (1994) have shown that FGF-2 soaked beads can induce formation of nearly normal limbs from wing buds which have had their AER removed. Furthermore, Savage *et al.* (1993) have suggested that curvature in the dorso-ventral plane of the wing bud is a result of higher expression of FGF-2 in the AER and dorsal ectoderm as compared to the ventral and that FGF-2 may serve to help suppress differentiation of mesodermal cells as well as promote their proliferation. It has also been shown that retroviral expression of FGF-2 in chick mesodermal cells grafted to different regions of the chick wing bud produced duplications of wing structures (Riley *et al.*, 1993). Moreover, FGF-2 is capable of inducing digit regeneration in the embryonic chick (Taylor *et al.*, 1994).

Statement of Purpose

That much limb mesoderm is susceptible to cell death under certain conditions *in vivo* has been shown by removal of the AER and underlying mesoderm (Rowe *et al.*, 1982). However, until now, with respect to the chick wing bud, only

regions of programmed cellular death (MacCabe *et al.*, 1991) and subridge mesoderm (MacCabe, 1993) have been shown to die *in vitro* in organ culture. The present investigation was undertaken in order to determine whether regions on the anterior and posterior flanks of the OP, which are not programmed to die *in vivo*, are susceptible to apoptosis *in vitro* and, if so, whether they can be "rescued" by FGF-2.

2. METHODS

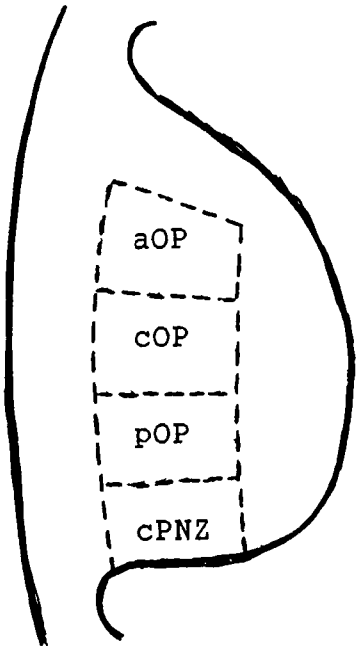
Fertile White Leghorn chicken eggs from the University of Tennessee, Knoxville, poultry farm, were stored from zero to six days at 12-15°C. After removal of 2ml of albumin, each egg was windowed (as in Rugh, 1948; with the major exception being that a sheet of wax was affixed to serve as a window) and incubated until it reached stage 21 (Hamburger and Hamilton, 1951). To expose the embryo, chorionic and amniotic membranes were gently torn open and pulled back with forceps. Tissues were removed from both wing buds of each embryo with a tungsten filament taking care not to cut through the ventral surface of the limb bud. Tissues were transferred by pasteur pipette into one of two dishes containing chilled phosphate-buffered saline (PBS) before being utilized in various assays. Experimental and control cultures were kept genetically indentical for portions of this study involving ^{51}Cr release assays and transmission electron microscopy (TEM). This was accomplished by placing tissue from the right wing bud of an embryo in one dish and placing the contralateral piece in the other. In addition, with each successive embryo the placement of tissue in dishes was reversed so that a dish containing six pieces of tissue contained three from right wing buds and three from left wing buds while still possessing no two from the same

embryo. All assays consisted of an experimental treatment coupled with a control.

^{51}*Cr Release Assay*

This assay was performed for four regions of tissue (illustrated in Figure 1) removed from the stage 21 wing bud (Hamburger and Hamilton, 1951): anterior to the opaque patch (aOP), posterior to the opaque patch (pOP), containing the prospective opaque patch (cOP), and containing the prospective posterior necrotic zone (cPNZ). The assay had been performed previously on the latter two regions for certain treatments (MacCabe *et al.*, 1991; for example) and was performed for some treatments in this study for the purpose of comparing the amount of cellular death between regions. Collected tissues from each dish of PBS were transferred by micropipette into dishes containing 3ml of minimum essential medium plus penicillin, streptomycin, and 10% calf serum (MEMps + 10% CS) taking care to transfer as little PBS as possible. Tissues were then transferred along with 200ul of medium into wells of multiwell plates. Each well, whether experimental or control, contained four to six pieces of tissue. Tissues were oriented so that mesodermal cells contacted both bottom and side of the well in addition to mesoderm of adjacent blocks of tissue. A 20ul volume of $Na_2{}^{51}CrO_4$ solution (1mCi, 37MBq/ml) was then added to each

Figure 1. An outline drawing representing the dorsal view of a chick embryo's right wing bud at stage 21 (based on Hamburger and Hamilton, 1951). Regions of tissue utilized are indicated as follows: aOP = anterior to the opaque patch; cOP = containing the prospective opaque patch; pOP = posterior to the opaque patch; cPNZ = containing the prospective posterior necrotic zone.


aOP
cOP
pOP
cPNZ

well. Tissues were incubated at 37.5°C in a 5% CO_2 atmosphere for six hours. Following this incubation, each well was rinsed six times by removal of 200ul of medium and replacement with 200ul of fresh medium. The last addition of medium differed between experimental and control wells. Control wells recieved 200ul of MEMps + 10% CS. Experimental wells used for FGF-2 treatments were filled with 200ul of 200ng/ml FGF-2 (or 10ng/ml FGF-2 as dictated by the experiment) in MEMps + 10% CS. Whereas experimental wells used for cyclohexamide (CHX) treatments received 200ul of MEMps + 10% CS and 2ul of CHX stock solution (1mg/1ml in PBS or 0.1mg/ml in PBS as dictated by the experiment). Experimental and control tissues were then incubated at 37.5°C in a 5% CO_2 atmosphere for 15 hours. Afterward, medium from each well (now containing chromium isotope released from cells by cell death) was transferred to its own scintillation vial. Then, 200ul of 1.0% Triton X-100 in PBS (a solution used to lyse cells) was micropipetted into wells, each of which still contained incubated tissues. After 10 minutes, lysate was collected from each well and was transferred to its own scintillation vial. The amount of isotope in each vial (two vials per well) was then counted by liquid scintillation. Percentage of chromium released was then calculated for each well by dividing the release value by the sum of the released value and the lysate value and multiplying by 100. This value (percent released; %R)

indicates the percentage of isotope released from tissues in a given well and can be used for comparing the amount of cell death between experimental and control wells of each experimental repetition. This assay was also performed for treatment of aOP tissue with 10ng/ml FGF-2 and 1ug/ml CHX in combination by addition of CHX stock solution after filling the experimental well with 200ul of 10ng/ml FGF-2 in MEMps + 10% CS.

For a final series of ^{51}Cr release assays utilizing aOP tissue a heparin sepharose column was prepared. A measure of 1.5 grams of heparin sepharose CL-6B beads was hydrated with 70ml distilled water (dH_2O) over the span of 30 minutes while agitating intermittently. A portion of hydrated beads was gently poured into a column and rinsed with small volumes (approximately 5ml each) of dH_2O until the column stabilized at a volume of approximately 6ml. The resultant column height was greater than 7ml. Calf serum was introduced just above the upper surface of the column and beneath approximately 2ml of dH_2O. A volume of 2ml dH_2O was maintained over the column throughout the process by slow addition of calf serum at the top of the column. A volume of serum was collected at a flow rate of approximately 4.1ul/sec. and was sterilized by vacuum filtration. An appropriate volume of vacuum sterilized MEMps was added to this volume of serum that had been passed through the column (hsCS) so that the resultant volume contained 10% hsCS. As

was done for the other ^{51}Cr release assays, tissue was incubated for a six hour uptake period in MEMps + 10% CS. Wells were then rinsed five times with 200ul of MEMps and the sixth addition of medium consisted of either 200ul MEMps + 10% hsCS (experimental wells) or 200ul MEMps + 10% CS (control wells).

Statistical Analysis

SAS statistical software was utilized for statistical analyses. A normal distribution of values within a population from which a sample is taken is one requirement for performing a t-test. Independence of samples is the other. Normality was assumed for the population and the manner in which ^{51}Cr release assays were performed insured independence of samples. The UNIVARIATE command was employed to perform paired, two-tailed t-tests to determine significance of the difference between each region's experimental and control %Rs for a given treatment. The ANOVA command was used to perform unpaired t-tests to compare %Rs between regions for each treatment that was performed on two or more regions of tissue. Within the ANOVA command, the Bonferonni t-test was chosen to control "experimentwise" error rate (at $p>0.05$ for all comparisons, together, within a group of comparisons). Groups were as follows: 10ng/ml FGF-2 treatment for aOP, cOP, pOP, and cPNZ

tissues; controls for the previous treatment; 200ng/ml FGF-2 treatment for aOP, cOP, and pOP tissues; and 10ng/ml FGF-2, 1ug/ml CHX, and combined treatments for aOP tissue.

Transmission Electron Microscopy

Subsequent to removal and collection in PBS, aOP and pOP tissues were transferred into their respective wells (determined by region) in a multiwell plate (four to six pieces per well). Wells contained 200ul of MEMps + 10% CS. Tissues were then incubated for six hours which corresponds with the uptake period in the ^{51}Cr release assays. Immediately afterward, medium was replaced in each well by removal of approximately 190ul of medium and addition of 200ul volume of fresh medium. Control wells were again filled only with MEMps + 10% CS; whereas, experimental wells contained either 200ng/ml FGF-2 or 10ug/ml CHX within the medium. Tissues were then incubated at 37.5^{0}C in a 5% CO_2 atmosphere for 15 hours. After being gently loosened with a tungsten filament and transferred to small glass containers, tissues were fixed in 3% Glutaraldehyde in 0.1M Phosphate buffer (pH 7.2) and rinsed three times in 0.1M Phosphate buffer. Tissues were post fixed for one hour with 2% OsO_4 in 0.1M Phosphate buffer (pH 7.2), dehydrated by a graded ethanol series, and embedded in Epon LX 112. Blocks were thin sectioned. Sections were post-stained with uranyl

acetate and lead citrate and observed using a Hitachi H-600 transmission electron microscope.

Gel Electrophoresis

Regions of tissue were removed and incubated for 21 hours (typically 20 to 50 pieces at a time) under either experimental (200ng/ml FGF-2 or 10ug/ml CHX in MEMps + 10% CS) or control (MEMps + 10% CS) conditions. Tissues were then stored at -20°C in nuclear isolation buffer (50mM Tris, 25mM KCl, 7.8mM MgOAc $\cdot 4H_2O$, 35mM sucrose, pH 7.6). When approximately 200 pieces of the region per treatment had been collected, tissues were thawed, consolidated, rinsed in PBS and homogenized in nuclear isolation buffer. Tissues were centrifuged at 12,000 rpm for four minutes, and the pellets were resuspended in extraction buffer (150mM NaCl, 1M Tris, 100mM EDTA) plus 7.5ul of 5M $NaClO_4$ and 7.5ul of 10% SDS stock solutions. Extraction was performed using extraction buffer saturated phenol and chloroform. DNA was precipitated overnight at -20°C in ethanol and NaOAc. A minimum of one day later, DNA was removed from cold storage and centrifuged at 12,000 rpm for 30 minutes. The resultant pellets were rinsed in 80% ethanol. Pellets were dried by vacuum centrifugation, resuspended in TE (100mM Tris, 10mM EDTA) and refrigerated overnight at -20°C. RNases A and T-1 were added, and DNA was incubated at 37.5°C in a water bath

for 30 minutes. Then, loading dye (bromophenol blue, 680mM sucrose, 10mM Tris) was added. DNA was then loaded into wells of a 1.5% agarose gel impregnated with 5ul of ethidium bromide stock solution (10mg/ml) that was immersed in TBE (2.5mM NaOH, 89mM Tris, 89mM Boric acid, 2mM EDTA, 25ul ethidium bromide stock solution). Typically, three lanes were used in each gel: tissues incubated in MEMps + 10% CS, experimental treatment in MEMps + 10% CS, and the Promega G1741 marker. A 73 volt current was passed through the gel for two to three hours; after which, the gel was viewed and photographed in ultraviolet light.

3. RESULTS

In Vitro Apoptosis

As can be seen in Figure 2, incubation of various tissues in MEMps + 10% CS (control condition) resulted in average %R values in excess of 40%. When average %R values for the four regions of tissue were compared to one another, no significant difference was found between cOP, pOP and cPNZ tissues or aOP and pOP tissues. However, significant differences were detected between aOP tissue and cOP and cPNZ tissues. Widespread cellular debris visible in micrographs of aOP and pOP tissues incubated under control condidtions (Figures 3A, and 4A, respectively) indicated that *in vitro* cell death had occurred. Moreover, highly pycnotic condition of much of the debris suggested that the mode of cell death might be apoptosis. Oligonucleosomal-sized fragmentation of DNA from aOP and pOP tissues incubated under control conditions (Figures 5, and 6, respectively) confirmed the occurrence of apoptosis. Laddering was evident at multiples of approximately 169 base pairs.

Treatment with 10ug/ml CHX (a protein synthesis inhibitor) resulted in significant inhibition of cell death

Figure 2. Average percent release (%R) values for *in vitro* culture of tissues incubated under control conditions. Controls represented are those that were coupled with 10ng/ml FGF-2 treatments. Regions of tissue are indicated as follows: aOP = anterior to the opaque patch; cOP = containing the prospective opaque patch; pOP = posterior to the opaque patch; cPNZ = containing the prospective posterior necrotic zone. Standard errors are indicated.

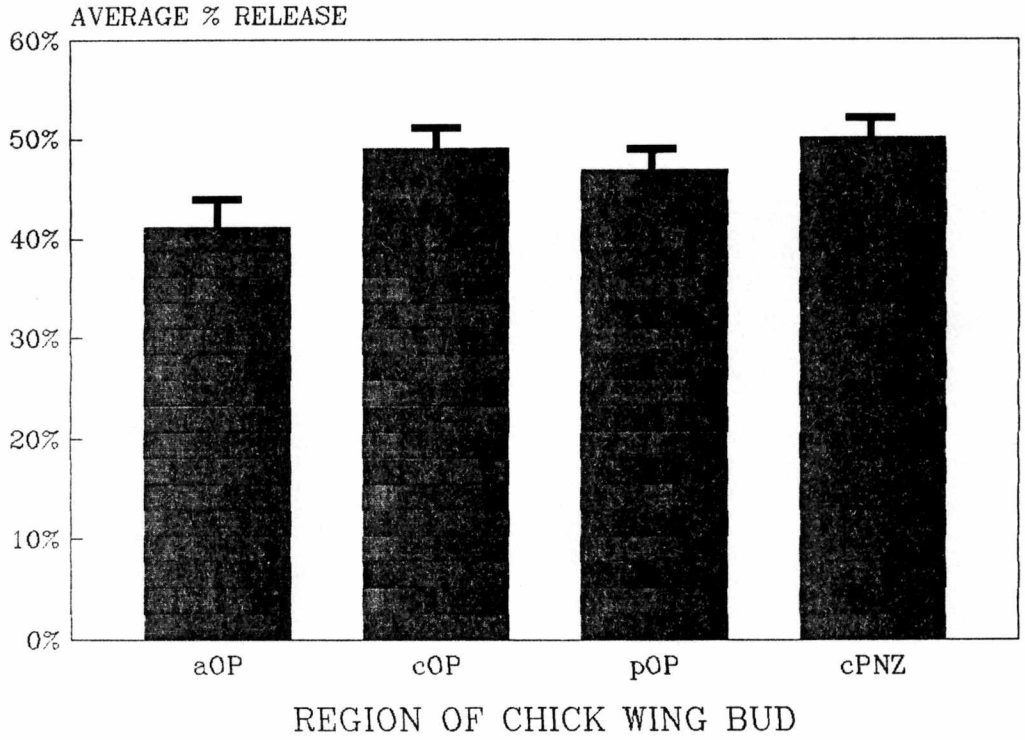

AVERAGE % RELEASE
60%
50%
40%
30%
20%
10%
0%
aOP
cOP
pOP
cPNZ
REGION OF CHICK WING BUD

Figure 3. Transmission electron micrographs of tissue anterior to the opaque patch (aOP) incubated in control medium and in medium containing CHX. Cultured *in vitro* in (A) MEMps + 10% CS or (B) 10ug/ml CHX in MEMps + 10% CS. CHX was added after six hours of incubation in MEMps + 10% CS and continued for the following 15 hours. Note prolific pycnotic debris (d) present among and within cells of untreated tissue. Bar lengths = (A) 5um and (B) 4um.

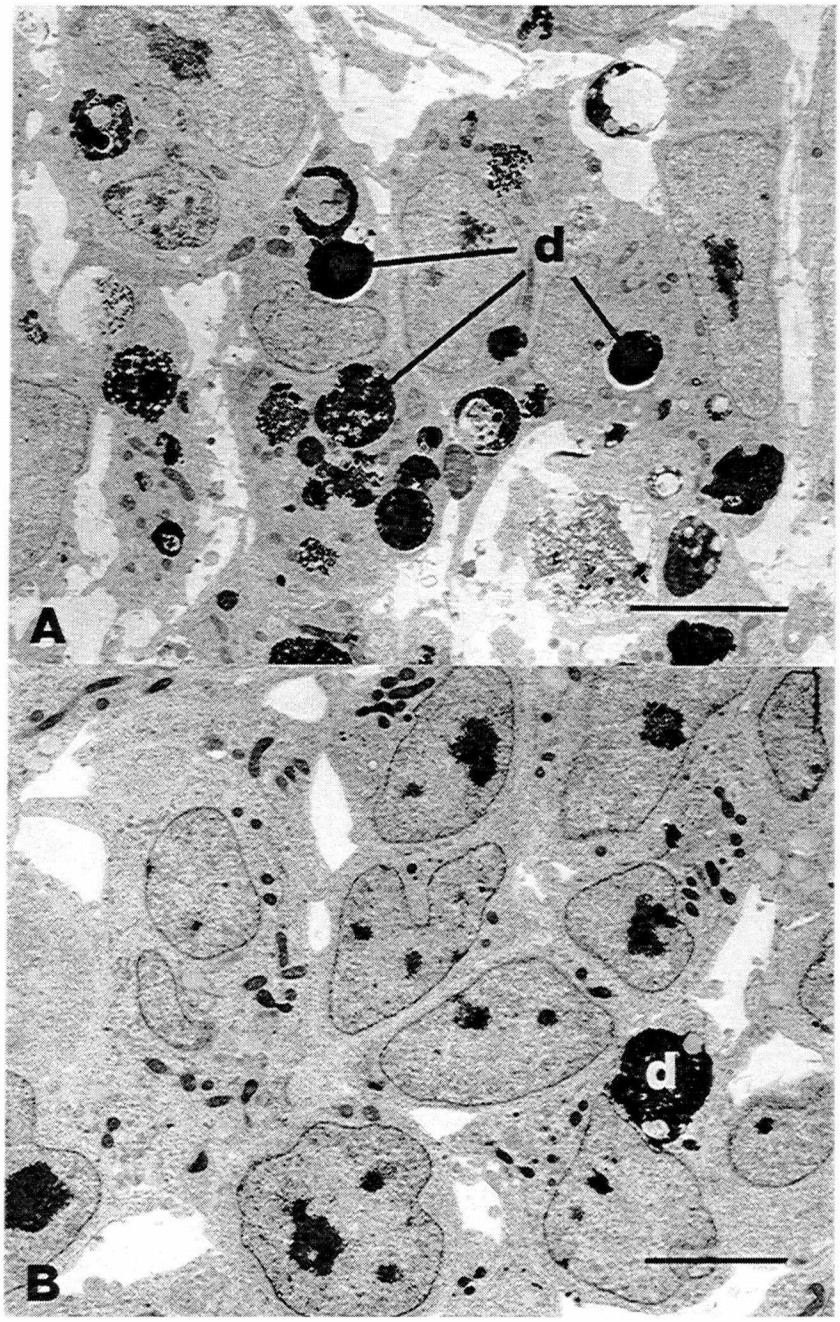

d
A
d
B

Figure 4. Transmission electron micrographs of tissue posterior to the opaque patch (pOP) incubated in control medium and in medium containing CHX. Cultured *in vitro* in (A) MEMps + 10% CS or (B) 10ug/ml CHX in MEMps + 10% CS. CHX was added after six hours of incubation in MEMps + 10% CS and continued for the following 15 hours. Note prolific pycnotic debris (d) present among and within cells of untreated tissue. Bar lengths = (A) 4um and (B) 4um.

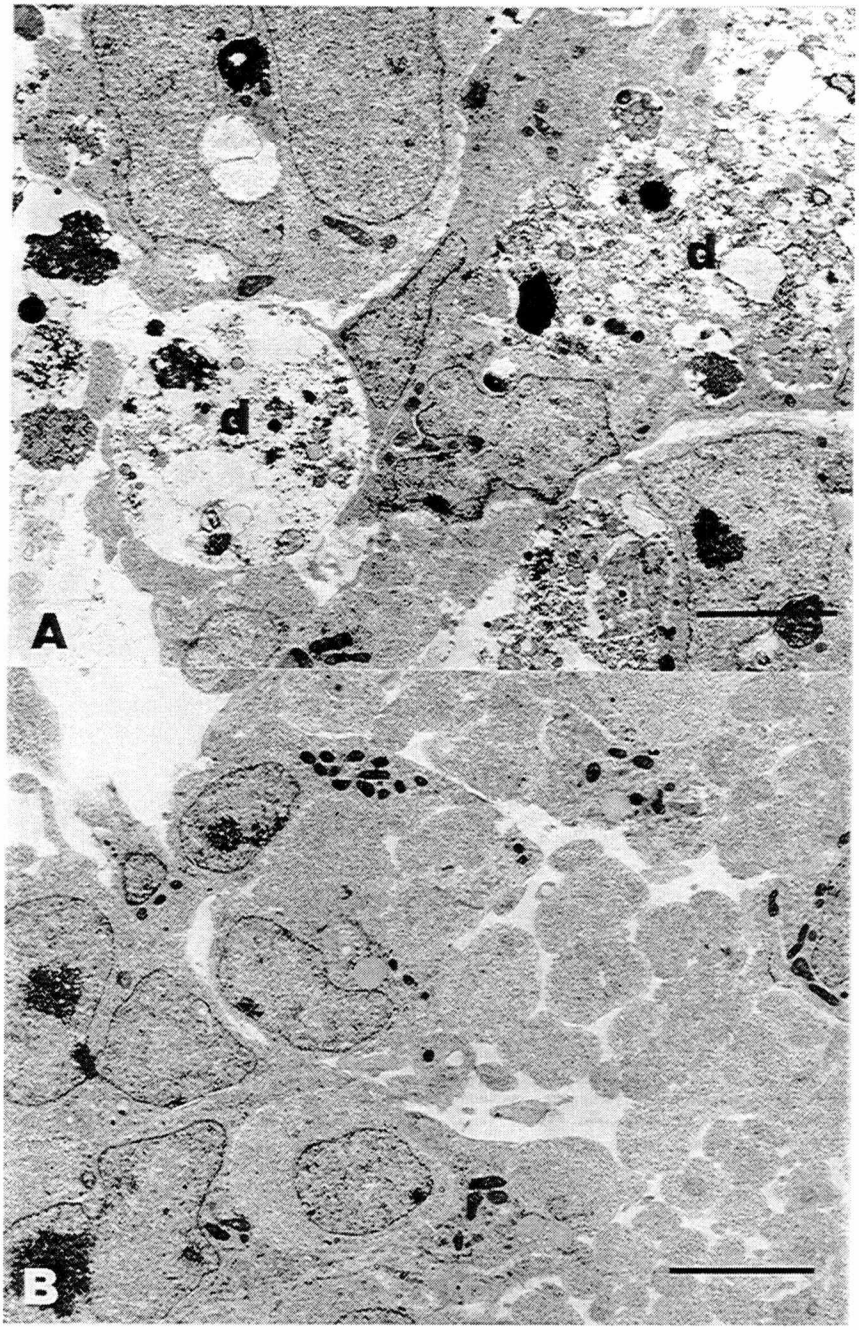

d
d
A
B

Figure 5. Electrophoresis of DNA extracted from tissue anterior to the opaque patch (aOP) cultured *in vitro* and run in 1.5% agarose gels. Panel A: lanes (1) Promega G1741 standard, (2) tissue incubated in MEM + 10% CS, and (3) tissue incubated in 10ug/ml CHX in MEM + 10% CS. Panel B: lanes (1) Promega G1741 standard, (2) tissue incubated in MEM + 10% CS, and (3) tissue incubated in 200ng/ml FGF-2 in MEM + 10% CS.

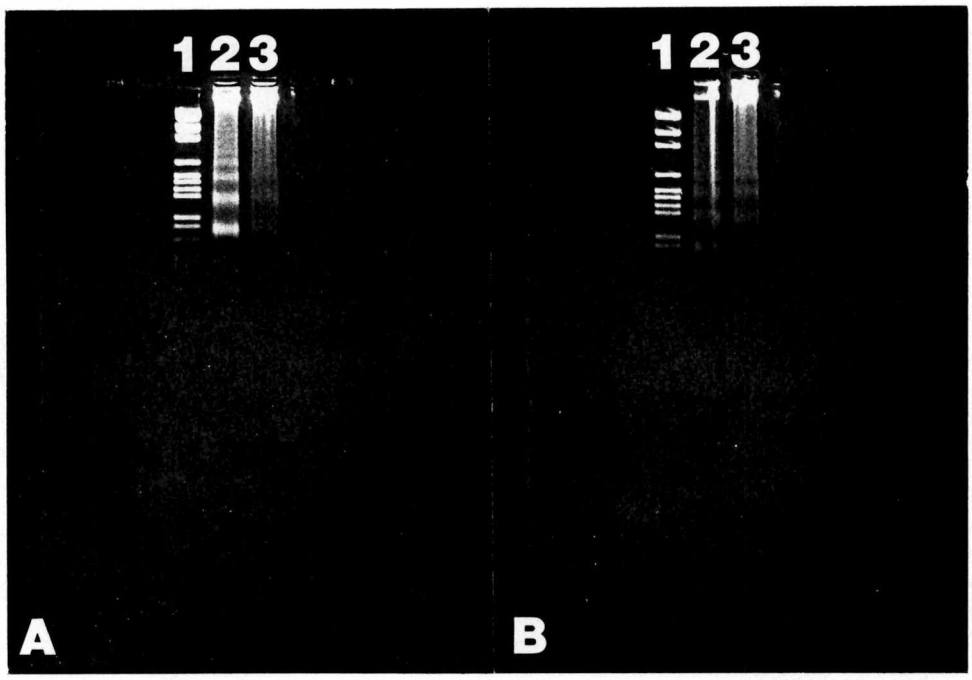
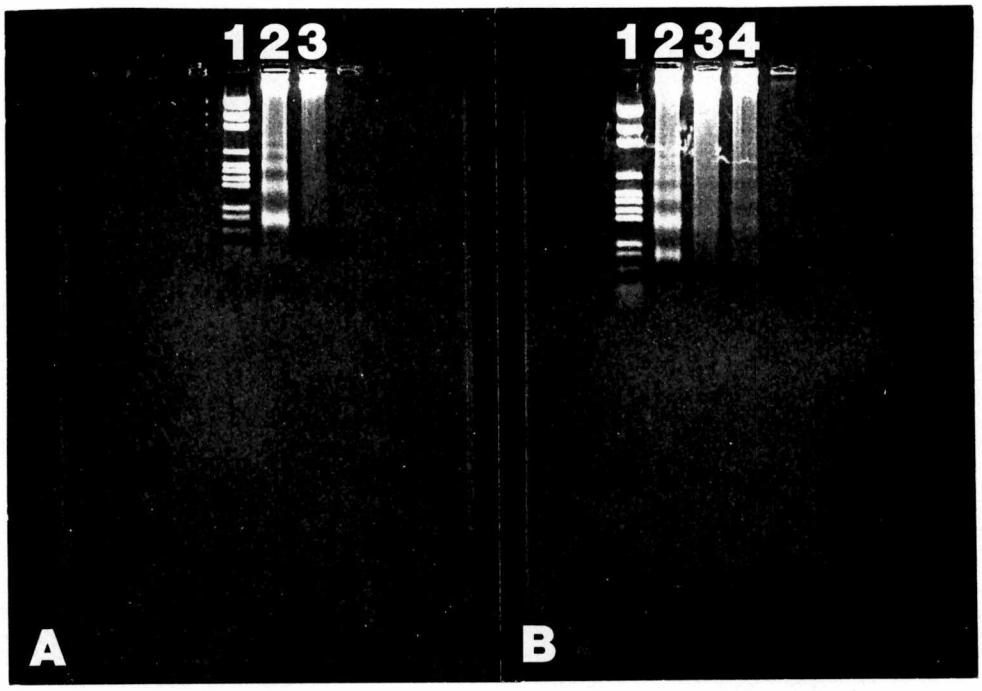

1 2 3
A
1 2 3
B

Figure 6. Electrophoresis of DNA extracted from tissue posterior to the opaque patch (pOP) cultured *in vitro* and run in 1.5% agarose gels. Panel A: lanes (1) Promega G1741 standard, (2) tissue incubated in MEM + 10% CS, and (3) tissue incubated in 10ng/ml CHX in MEM + 10% CS. Panel B: lanes (1) Promega G1741 standard, (2) tissue incubated in MEM + 10% CS, (3) tissue incubated in 10ng/ml CHX in MEM + 10% CS, and (4) tissue incubated in 200ng/ml FGF-2 in MEM + 10% CS.


1 2 3
1 2 3 4
A
B

(Figure 7; Table 1, Appendix A),* as demonstrated by lower %R values relative to incubation under control condition. This concentration of CHX also significantly inhibited cell death in cOP tissue (Figure 7; Table 1, Appendix A). Inhibition of apoptosis in aOP and pOP tissues was confirmed by TEM (Figures 3B, p.23 and 4B, p.25). As demonstrated by electrophoretic gels, treatment with CHX resulted in inhibition of laddering of DNA of both aOP and pOP tissues (Figures 5A, p.27 and 6A, p.29). This further implicated apoptosis. Any laddering that is present in lanes corresponding to DNA from CHX treated tissues was very faint. Since CHX was added at the beginning of incubation periods for gels, light laddering visible in some gels suggests that a concentration of 10ug/ml CHX may not be capable of entirely inhibiting apoptosis.

Response to FGF-2

Treatment with 200ng/ml FGF-2 (Figure 7) also resulted in reduced occurrence of cell deaths. Differences were determined to be significant (Table 1, Appendix A). Scant amount of cellular debris in micrographs of aOP and pOP tissues under this treatment condition (Figures 8, and 9, respectively) also indicates that apoptosis in these tissues

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* For the table of probability values, see Appendix A.

Figure 7. Average percent release (%R) values for *in vitro* culture of tissues anterior to the opaque patch (aOP), containing the prospective opaque patch (cOP), and posterior to the opaque patch (pOP) treated with either 200ng/ml FGF-2 or 10ug/ml CHX. Dark bars represent treatment with FGF-2. Vertically striped bars represent treatment with CHX. Empty bars represent controls (MEM + 10% CS, only). Standard errors are indicated.

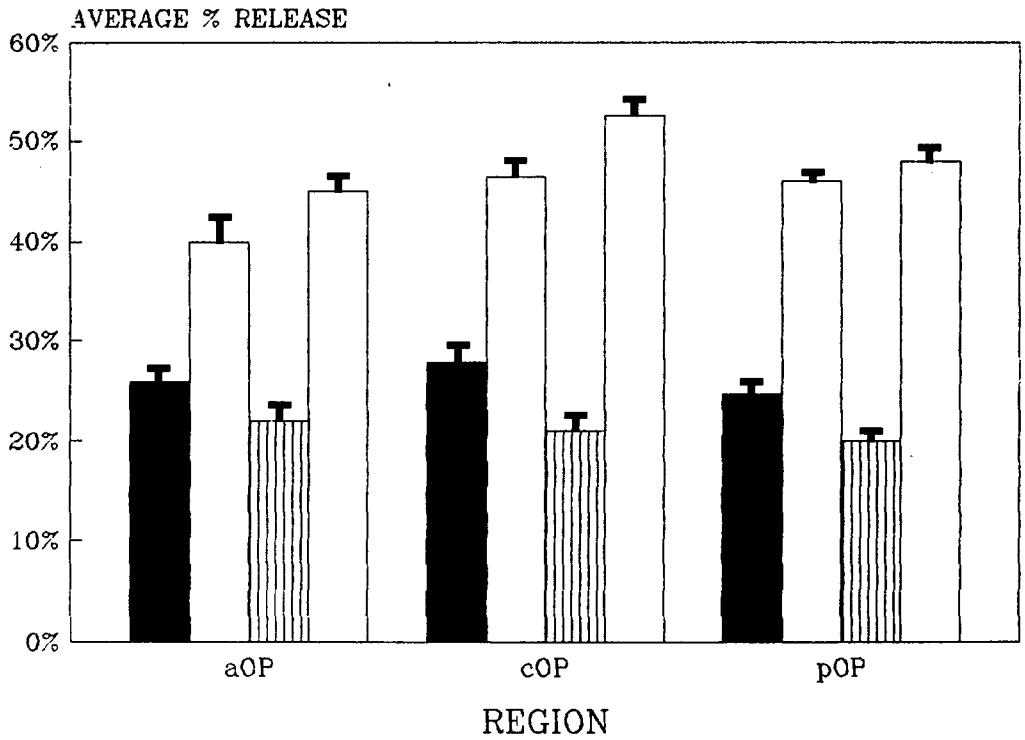

AVERAGE % RELEASE
60%
50%
40%
30%
20%
10%
0%
aOP
cOP
pOP
REGION

Figure 8. Transmission electron micrographs of tissue anterior to the opaque patch (aOP) incubated with FGF-2 in the medium. Cultured *in vitro* in 200ng/ml FGF-2 in MEM + 10% CS. FGF-2 was added after six hours of incubation in MEM + 10% CS and continued for the following 15 hours. Panel A shows the usual debris free appearance of treated tissue. Panel B shows the typical form and lack of abundance of debris (d) when detected in treated tissue. Bar lengths = (A) 4um and (B) 4um.

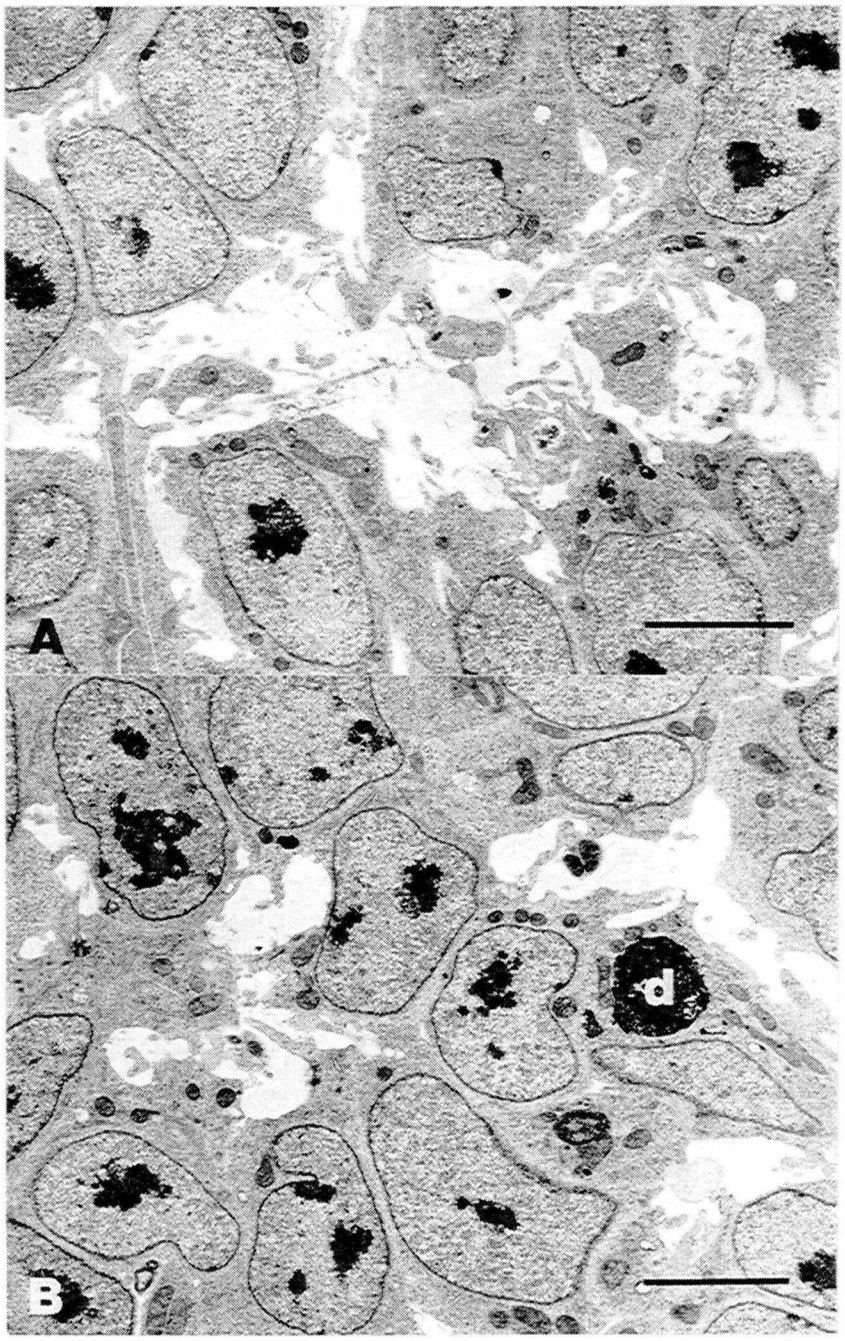
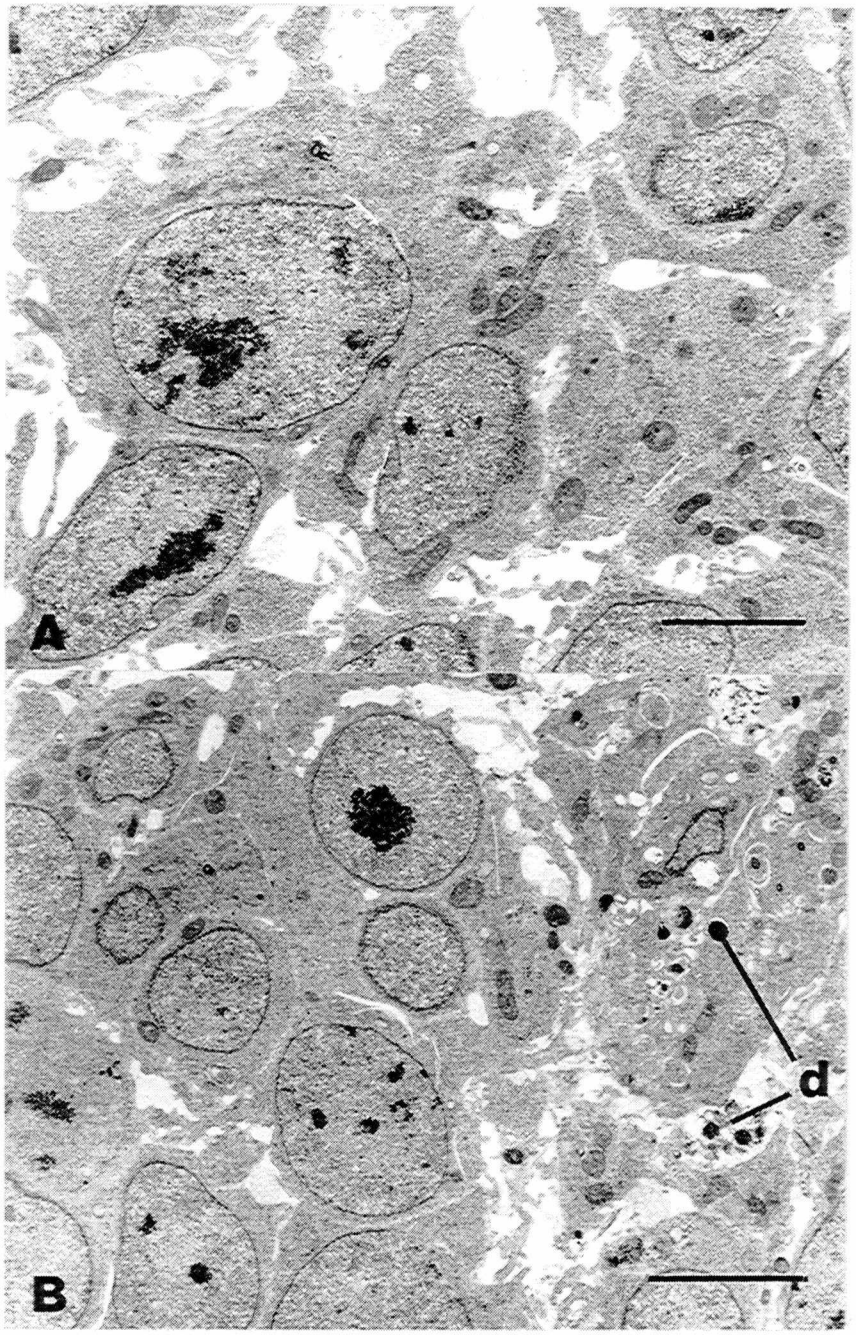

A
d
B

Figure 9. Transmission electron micrographs of tissue posterior to the opaque patch (pOP) incubated with FGF-2 in the medium. Cultured *in vitro* in 200ng/ml FGF-2 in MEM + 10% CS. Treatment was added after six hours of incubation in MEM + 10% CS and continued for the following 15 hours. Panel A shows the usual debris free appearance of treated tissue. Panel B shows the typical form and lack of abundance of debris (d) when detected within treated tissue. Bar lengths = (A) 4um and (B) 5um.


A
B
d

can be inhibited by this concentration of FGF-2. Surprisingly, treatment with 200ng/ml FGF-2 did not eliminate DNA laddering (Figures 5B, p.27 and 6B, p.29). However, a gel where pOP DNA collected from both FGF-2 and CHX treatments was run concurrently (in separate lanes) exhibited differences in laddering intensity. Though more intense than in the lane corresponding to CHX treatment, laddering in the lane corresponding to FGF-2 treatment was slightly less intense than that in the lane corresponding to control treatment (Figure 6B, p.29).

As indicated by ^{51}Cr release assay, treatment with 10ng/ml FGF-2 also resulted in significant "rescue" for each of the four regions of tissue (Figure 10; Table 1, Appendix A). Comparison of average experimental %R values between the four regions for 10ng/ml FGF-2 treatment showed no significant difference between aOP and pOP tissues, pOP and cOP tissues, or cOP and cPNZ tissues. All other comparisons resulted in significant differences. However, when treated with 200ng/ml FGF-2 no significant difference was revealed between aOP, cOP, and pOP tissues. The cPNZ tissue was excluded from treatment with 200ng/ml FGF-2.

Combined and hsCS Treatments

Figure 11 shows the effect of various experimental treatments (10% hsCS, 10ng/ml FGF-2, 1ug/ml CHX and

Figure 10. Average percent release (%R) values for *in vitro* culture of tissues treated with 10ng/ml FGF-2. Regions of tissue are indicated as follows: aOP = anterior to the opaque patch; cOP = containing the prospective opaque patch; pOP = posterior to the opaque patch; cPNZ = containing the prospective posterior necrotic zone. Dark bars represent treatments. Empty bars represent controls (MEM + 10% CS, only). Standard errors are indicated.

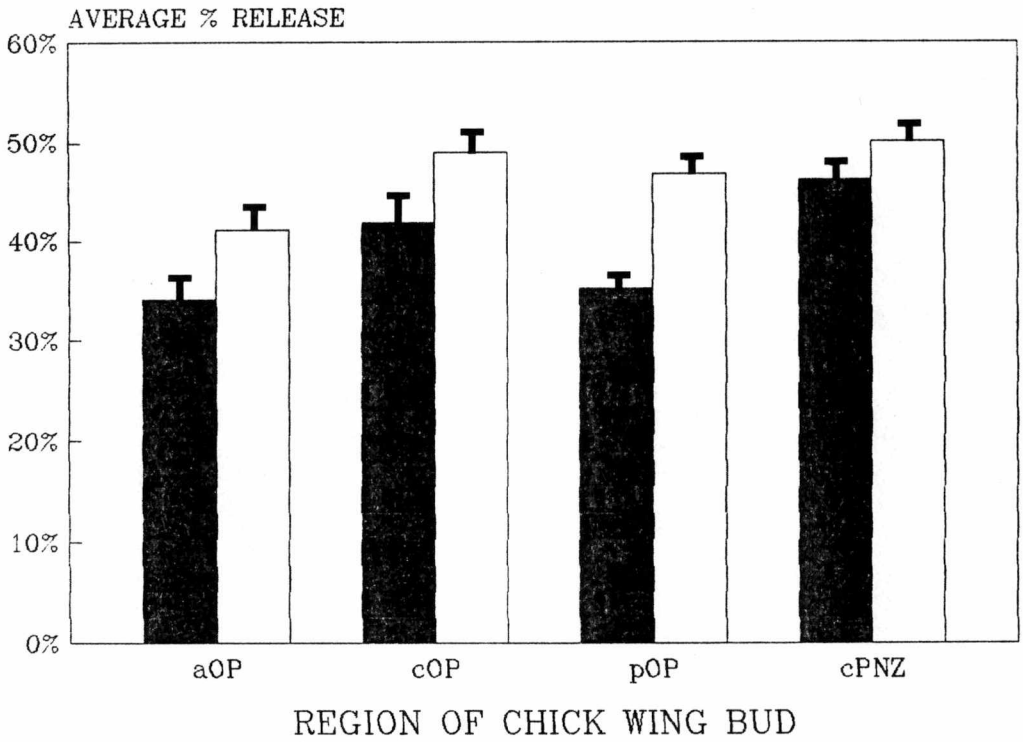
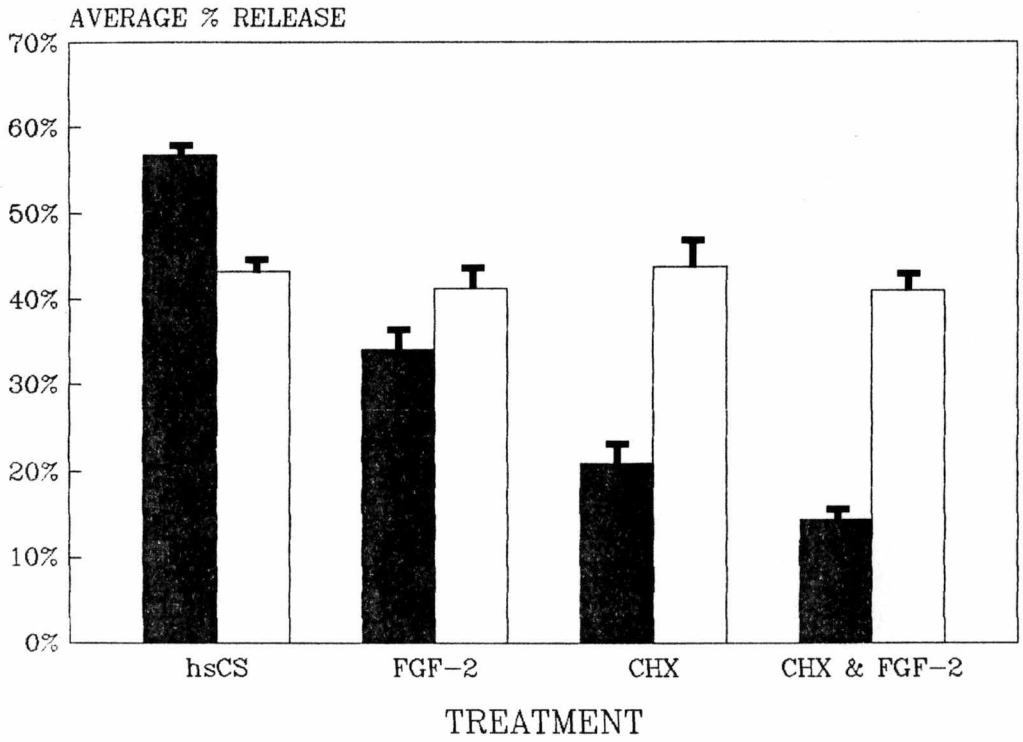

AVERAGE % RELEASE
60%
50%
40%
30%
20%
10%
0%
aOP
cOP
pOP
cPNZ
REGION OF CHICK WING BUD

Figure 11. Average percent release (%R) values for *in vitro* culture of tissue anterior to the opaque patch (aOP) in MEM + 10% hsCS or in MEM + 10% CS containing either 10ng/ml FGF-2, 1ug/ml CHX, or both. Dark bars represent treatments. Empty bars represent controls (MEM + 10% CS, only). Standard errors are indicated.


AVERAGE % RELEASE
70%
60%
50%
40%
30%
20%
10%
0%
hsCS
FGF-2
CHX
CHX & FGF-2
TREATMENT

10ng/ml FGF-2 plus 1ug/ml CHX) on *in vitro* cell death in aOP tissue. FGF-2, CHX, and FGF-2 plus CHX treatments all significantly reduced average %R values (Table 1, Appendix A) and, so, presumably inhibited cell death. Furthermore, their respective average experimental %R values were determined to be significantly different from one another. This indicates that the amount of "rescue" differed between treatments. Use of FGF-2 and CHX in combination enhanced cellular survival more than either treatment alone. Although FGF-2 appears to be capable of influencing nuclear transcriptional events (Hawker and Granger, 1994; Bouche *et al.*, 1994; Amalric *et al.*, 1994), the reduced average %R value resulting from combined treatment may suggest that FGF-2, in part, inhibits PCD by a method or methods that do not involve *de novo* protein synthesis. However, these data do not eliminate other explanations for enhanced "rescue" by combined treament. Inhibition of some CHX induced death by FGF-2 may be involved or, perhaps, incomplete inhibition of protein synthesis by CHX may have allowed for expression of genes whose transcription had been upregulated by FGF-2. The hsCS treatment of aOP tissue was not included in the above statistical analysis since it significantly increased cell death (Table 1, Appendix A) and therefore was obviously divergent from "rescuing" treatments in its effect.

4. DISCUSSION

In Vitro Apoptosis in aOP and pOP Tissues

Apical mesoderm incubated with the AER intact does not exhibit a considerable amount of cell death in organ culture. Average %R values generated under such a condition have been determined to be approximately 29% (MacCabe *et al.*, 1991) and 36% (MacCabe, 1993). A comparison of these values with average %R values generated for the four regions in this study (Figure 2, p.21) indicated that cell death had likely occurred in aOP, cOP, pOP and cPNZ tissues when incubated under control conditions. The occurrence of cell death was confirmed for aOP and pOP tissues by TEM.

Though cells within aOP and pOP regions do not undergo PCD *in vivo,* these tissues exhibited considerable amounts of cell death *in vitro* that possesses characteristics of apoptosis such as production of oligonucleosomal-sized fragments of DNA in dying tissue and "rescue" by inhibition of protein synthesis. This seems to contradict findings of Fallon and Saunders (1968) who determined that tissue removed from the dorsal wing bud and placed into culture showed no significant evidence of cell death. The discrepancy between the present study and the research by Fallon and Saunders might be explained, in part, by utilization of different culture media. In a study of the

PNZ cultured *in vitro* in MEMps + 10% CS by MacCabe and Brewton (1984), morphological evidence of cellular death began to be apparent in the PNZ at late stage 22 or late stage 23. However, in a previous study performed by Fallon and Saunders (1968) when cultured *in vitro* in Eagle's basal medium + 10% horse serum, 1% glutamine, 1% embryo extract, penicillin and streptomycin, morphological signs of death became apparent at late stage 23 or early 24. Fibroblast growth factor is known to be found throughout much of embryonic development (Seed *et al.*, 1988). It may be the presence of this and other factors in the embryo extract used by Fallon and Saunders (1968) which is responsible for disagreement between their study and this one concerning *in vitro* death or survival of wing mesenchyme from regions that do not exhibit PCD *in vivo*.

That cells within tissues anterior and posterior to the opaque patch do not exhibit considerable cell death *in vivo* yet are capable of undergoing apoptosis in organ culture has implications concerning the manner in which limb bud cells are directed toward the apoptotic fate *in vivo*. It seems to suggest that maintenance or death of these cells in the developing limb bud might best be attributed to an external factor or factors that are accessible in the limb bud but are not *in vitro*. In apparent contrast to this, Fallon and Saunders (1968) have shown that PNZ tissue exhibits cell death on schedule even when removed and grafted to somites

or cultured *in vitro,* results which imply that developmental fate and its timing is determined by cells within a region of developmental death. However, they also showed that association of the PNZ with other wing bud tissue *in ovo* or *in vitro* prevented cell death in the PNZ which suggests that adjacent tissues influence the PCD process. It appears that a factor or factors which induce PCD in the PNZ may be located in nearby ectoderm since removal of ectoderm inhibits *in vivo* cell deaths therein (Brewton and MacCabe, 1988). However, in the same study, removal of ectoderm did not inhibit *in vitro* cell death. This suggests that an *in vivo* survival factor is also present within the developing limb bud. Moreover, absence of this survival factor *in vitro* appears sufficient to induce cell death even after removal of a possible death factor which may emanate from ectoderm adjacent to the PNZ.

FGF-2 Treatment and DNA Fragmentation

Laddering of DNA despite treatment with FGF-2 seems to suggests that FGF-2 does not considerably inhibit apoptosis in aOP and pOP tissues. This, however, contradicts both TEM evidence which suggests "rescue" by FGF-2 since tissues incubated under such treatment contained neither cells exhibiting apoptotic morphology nor much cellular debris and results from the ^{51}Cr release assays. Yet, this discrepancy

may be reconciled.

For instance, a prolonged period of tissue collection for gels during which pieces of tissue were kept in chilled PBS for up to six hours may have resulted in initiation of the apoptotic program. It is possible that a prolonged period of low temperature produced damage that subsequently caused many cells in the tissues to undergo apoptotic death when brought to incubating temperature (Clavien *et al.*, 1992). It has been shown that exposure of tumor cells to cold (used to disrupt microtubules) can induce apoptosis (reviewed in Liepins and Bustamante, 1994). Damage might occur to an FGF-2 internalization pathway or pathways. It has been found that cell surface proteoglycans needed for FGF-2 signal transduction co-localize with the actin cytoskeleton of the cell (Fernández-Borja *et al.*, 1995). However, it was also shown that depolymerization of microtubules did not alter distribution of bound FGF-2 on the cell surface. Therefore, if cold-induced damage is responsible for laddering in FGF-2 lanes due to disruption of FGF-2 signal transduction, interference likely occurs in a manner which involves neither redistribution of receptors nor their removal from the cell surface. Since *de novo* protein synthesis is required for apoptosis to occur, one would not expect to see such laddering in the lanes of DNA corresponding to CHX treatment as long as the concentration of CHX used is high enough to greatly inhibit protein

synthesis, despite a prolonged collection period. This is consistent with what was observed in electrophoretic gels.

Alternatively, a concentration of 200ng/ml FGF-2 may not be capable of completely inhibiting apoptosis within these embryonic tissues. Support for this argument is found in results of the ^{51}Cr release assays. Addition of 10ng/ml FGF-2 plus 1ug/ml CHX to medium resulted in a lower average %R value for aOP tissue than did a concentration of 200ng/ml FGF-2 (Figures 10, p.39 and 7, p.32, respectively). In addition, some (though little) cellular debris was visible by TEM in FGF-2 treated tissues. It has been suggested by Hase *et al.* (1994) that two distinct signal transduction pathways may be involved in suppression of apoptosis by maintaining a critical level of phosphorylated tyrosines within a cell. One pathway might serve as a stimulator of tyrosine kinase activity while another acts as an inhibitor of tyrosine phosphatase activity. The two (or more) pathways may not have been maximally activated in this study. Additionally, It may not be possible for FGF-2 to "rescue" all cells that are susceptible to apoptosis within a region of tissue despite the presence or absence of additional factors. Aono and Ide (1988) have shown that, in the chick limb bud, there is an overall trend of responsiveness to FGF which decreases posteriorly. Seed and Hauschka (1988) have shown that myoblasts of chick embryos exhibit differences in responsiveness to FGFs, and their differentiation (though

postponed) can not be prevented by FGFs. In addition, a recent study by Noveroske and MacCabe (1995) has shown that the progression of apoptosis differs between OP and PNZ tissues after brief exposure to FGF-2. Therefore, it seems that the ability of FGF-2 to influence development varies between regions of the limb bud. Perhaps regions of abundant developmental cell death contain large subpopulations of cells that are not as responsive to FGF-2 as are dominant subpopulations of cells in regions that do not exhibit considerable cell death during development. Results of treatment with 10ng/ml FGF-2 support this supposition. The aOP and pOP tissues were found to be more responsive to FGF-2 than were tissues located to their immediate posteriors (cOP and cPNZ tissues, respectively) which contain regions wherein an ample amount of cell death is evident *in vivo*. Lack of significant difference in responsiveness to FGF-2 between cOP and pOP tissues seems to confound this speculation; yet, an explanation for this incongruity may exist. An overall gradient of responsiveness to FGF-2 as detected by Aono and Ide (1988) might coexist in the limb bud with regions wherein subpopulations of cells predominate which are less responsive to FGF-2 than are, on average, cells of adjacent proximal and distal regions. It may be that limb bud mesenchyme consists of cell types that differ in responsiveness to FGF-2 (or FGFs in general) and that what determines whether a region displays considerable

developmental cell death or not depends on the prevalence of one cell type over another within a given region's cell population. Therefore, some (though infrequent) apoptosis might occur in tissues outside of the ANZ, OP, PNZ and INZ within the limb bud. This may be what is represented in the gels by laddering of DNA in lanes corresponding to 200ng/ml FGF-2 treatment.

Heparin Binding Factors and Cell Survival

Yonezawa *et al.* (1992) have shown that sera possess various heparin binding factors which stimulate mitosis. In the present study, the significantly higher average %R value (increase in cell deaths) for aOP tissue incubated with 10% hsCS instead of 10% CS in the medium indicates that removal of heparin binding growth factors from serum increases the occurrence of cell deaths. This implies that heparin binding factors, such as those within calf serum, are somewhat capable of reducing the incidence of cell death in aOP tissue. FGF-2 is one such factor that interacts with heparin in the extracellular matrix (ECM) prior to binding with high affinity cell surface receptors (reviewed in Klagsbrun and Baird, 1991). It is possible that heparin in the ECM might play a role in defining the degree that FGF-2 can influence cell proliferation and survival in different regions of the limb bud.

Summary and Conclusions

The results of this study illustrate that anterior and posterior chick wing bud tissues that elude the PCD process during development are susceptible to apoptosis in organ culture. This death is characterized by production of oligonucleosomal-sized lengths of DNA as well as inhibition of death and DNA fragmentation by treatment with an inhibitor of protein synthesis (CHX). The occurrence of apoptosis in these tissues in organ culture suggests that developmental cell death in some regions of the chick limb bud mesenchyme may result from a lack of access to a certain factor or factors. This may complicate some *in vitro* studies of apoptosis which, by nature, remove tissues from their native environment. In so doing, tissues may be removed from the influence of a factor or factors that must be present in order to make meaningful inferences concerning *in vivo* processes.

In addition, strong evidence from ^{51}Cr release assays and transmission electron micrographs indicates that the apoptosis observed in this study is inhibited by FGF-2. FGF-2 is known to be expressed in the chick limb bud (Muniam *et al.*, 1988; Savage *et al.*, 1993) and, along with others of the FGF family, is likely responsible for cell proliferation in the limb bud (reviewed in Tanaka and Gann, 1995). Therefore, the results of this study also suggest

that inhibition of apoptosis in most cells of aOP and pOP tissues *in vivo* may, in part, be attributable to FGF-2 present in the developing limb bud.

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APPENDICES

APPENDIX A

Table 1. Probability values for paired t-tests performed for ^{51}Cr release assays. Regions of tissue are indicated as follows: aOP = anterior to the opaque patch; cOP = containing the prospective opaque patch; pOP = posterior to the opaque patch; cPNZ = containing the prospective posterior necrotic zone. NP = experiment was not performed.

	aOP	cOP	pOP	cPNZ
200ng/ml FGF-2	0.0001	0.0003	0.0001	NP
10ng/ml FGF-2	0.0066	0.0004	0.0001	0.0001
10ug/ml CHX	0.0001	0.0001	0.0001	NP
1ug/ml CHX	0.0001	NP	NP	NP
10ng/ml FGF-2 & 1ug/ml CHX	0.0001	NP	NP	NP
Heparin Treated Serum (hsCS)	0.0001	NP	NP	NP

APPENDIX B

LIST OF ABBREVIATIONS AND SYMBOLS

AER	apical ectodermal ridge
ANOVA	analysis of variance
ANZ	anterior necrotic zone
aOP	anterior to the opaque patch
BMP	bone morphogenetic protein
bp	base pairs
C	celsius
CHX	cycloheximide
cm	centimeters
cOP	containing the prospective opaque patch
CO_2	carbon dioxide
cPNZ	containing the presumptive posterior necrotic zone
CS	calf serum
dH_2O	distilled water
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	(ethylene dinitrilo)-tetraacetic acid
EtBr	ethidium bromide
ETOH	ethyl alcohol
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
hsCS	calf serum subsequent to being passed through a heparin sepharose column

INZ	interdigital necrotic zone(s)
kbp	kilobase pairs
KCl	potassium chloride
M	moles
MBq	megabecquerels
mCi	millicuries
MEMps	minimum essential medium plus penicillin and streptomycin
$MgOAc \cdot 4H_2O$	magnesium acetate tetrahydrate
ml	milliliters
mM	millimoles
NaCl	sodium chloride
$NaClO_4$	sodium perchlorate
NaOAc	sodium acetate
NaOH	sodium hydroxide
$Na_2{}^{51}CrO_4$	chromium isotope salt
ng	nanograms
OP	opaque patch
OsO_4	osmium tetroxide
PBS	phosphate buffered saline
PCD	programmed cell death
PNZ	posterior necrotic zone
pOP	posterior to the opaque patch
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate

sec	second
TBE	tris-boric acid-EDTA solution
TE	tris-EDTA solution
TEM	transmission electron microscopy
TGF-*B*	transforming growth factor beta
ug	micrograms
ul	microliters
um	micrometers
ZPA	zone of polarizing activity
%R	percent release
^{51}Cr	chromium isotope 51

VITA

Paul Richard Ortstadt was born in Sacramento, CA on June 2, 1968. He attended public schools in Fishkill, NY, Scottsboro, AL and Knoxville, TN. He graduated from Farragut High School in June 1986. The following year, he enrolled in Roane State Community College; after which, he transferred to the University of Tennessee, Knoxville. He was received into the Phi Beta Cappa, Golden Key and Phi Cappa Phi honor societies, and he graduated in May 1992, magna cum lauda, with a Bachelor of Science degree in Zoology/Organismal and Systems Biology. In the remainder of the year he was employed by the Knoxville Recycling Coalition and then Advanced Sciences, Inc. In January of 1993 he reentered the University of Tennessee, Knoxville to pursue a Master of Science degree in Zoology. The degree was conferred December 1996.