To the Graduate Council:

I am submitting herewith a dissertation written by Steven W. Gay entitled "Embryonic Limb Outgrowth: Differentiation and Pattern Formation." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

Jeffrey A. MacCabe, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

EMBRYONIC LIMB OUTGROWTH: DIFFERENTIATION AND PATTERN FORMATION

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Steven W. Gay

June 1982

3060871

ACKNOWLEDGEMENTS

No scientific work is ever the product of only one person's efforts. For the work I report here, I would like to thank my major professor, Dr. Jeff MacCabe. His interest, encouragement and support made this work possible; his friendship made it worthwhile and enjoyable. The members of my committee helped make my work progress as smoothly as possible, and were understanding when things did not go smoothly. For this I am grateful to Dr. Jim Caponetti, Dr. M. L. Pan, Dr. Alex Shivers and Dr. Gary Whitson. I would also like to thank Dr. John Fallon and Dr. Robert Kosher for their comments and for their continuing interest in my work and ideas. Very special thanks goes to Maureen Allison and Al Cady for helping to put this volume together.

I have made many friends here in the last few years. To all my friends, thank you for everything.

ABSTRACT

The purpose of these experiments was to determine levels of commitment to differentiation and pattern formation in the developing chick limb bud. Two series of experiments were performed. In the first experiments, commitment to differentiation was assayed by testing the ability of limb bud tissue explants to form myotubes in culture. Strips of apical mesoderm, with or without the attached apical ectodermal ridge, and strips immediately proximal to these, were removed from stage 17 to 23 wing buds and placed in culture with a medium conducive to myogenesis. Relatively few cultures of apical explants contained myotubes when the explants were removed from stage 17 to 19 wing buds. No significant myogenesis was observed in cultures of tissue taken from wing buds of later stages. This result was not dependent on whether the apical ectodermal ridge was present or not. Cultures of more proximal explants generally contained myotubes by the second or third day of incubation. These results suggest that there is a special zone of subridge mesoderm that extends about 0.10–0.20 mm into the mesoderm. Cells in this zone are not able to differentiate when cultured.

In the second series of experiments, portions of stage 21 or 23 leg buds were grafted onto portions of stage 21 or 23 wing buds. Distal to proximal transplants created presumptive gaps in the proximodistal axis of the graft limb; proximal to distal transplants created presumptive extra segments. Regulation (correction of abnormalities in the presumptive proximodistal axis) was observed in distal to proximal graft limbs but not in limbs resulting from

proximal to distal grafts. The ability of host wing tissue to participate in regulation decreased at more proximal levels within the limb bud and with the age of the limb bud. These results, and those of previous investigations, suggest that proximodistal pattern is initially specified in subridge mesoderm and that the mesoderm becomes increasingly committed to this pattern specification at more proximal levels.

TABLE OF CONTENTS

CHAPTER PAGE

I. INTRODUCTION TO LIMB OUTGROWTH 1

II. DIFFERENTIATION 13

Introduction 13

Materials and Methods 15

Results 17

Discussion 19

III. PATTERN FORMATION 23

Introduction 23

Materials and Methods 26

Results 29

Discussion 35

LIST OF REFERENCES 46

APPENDIXES 52

APPENDIX I. FIGURES 53

APPENDIX II. TABLES 73

VITA 77

LIST OF FIGURES

FIGURE PAGE

1. Schematic Dorsal View of a Stage 21 Chick Wing Bud 54
2. Profiles of Chick Wing Buds During Distal Outgrowth 55
3. Chicken Limb Skeletal Structures, Showing Homologous Wing and Leg Proximodistal Segments 56
4. Fate Maps of Chick Wing Buds at Various Developmental Stages, Produced by Autoradiographic Labelling 57
5. Arrest of Outgrowth in Chick Wing Buds Produced by Excision of the Apical Ectodermal Ridge at Various Developmental Stages 59
6. Proximodistal Transplants as Performed by Summerbell, Lewis and Wolpert (1973) 60
7. Proximodistal Transplants as Performed by Kieny (1977) . . . 61
8. Apical and Proximal Explants Removed from Chick Wing Buds for Myogenesis Assay 62
9. Inverted Phase Contrast Photomicrographs of Cells from Apical and Proximal Explants After 72 Hours Incubation 63
10. Proximodistal Transplants as Reported in Materials and Methods, Chapter III, for Stage 21 Chick Limb Buds . . . 64
11. Proximodistal Transplants as Reported in Materials and Methods, Chapter III, for Stage 23 Chick Limb Buds . . . 65
12. Proximal to Distal Transplant Controls 66
13. Proximal to Distal Transplant Graft Limbs 67
14. Distal to Proximal Transplant Controls 69
15. Distal to Proximal Transplant Graft Limbs 71

CHAPTER I

INTRODUCTION TO LIMB OUTGROWTH

The combined mechanisms leading to the development of multicellular organisms are complex. Cells within a developing organism must divide, grow and differentiate in a temporally and spatially coordinated sequence. It is difficult to form a coherent understanding of an organism's development because of the many processes involved. This level of complexity can be reduced by analyzing developmental processes individually rather than simultaneously. Certain simpler organisms, or relatively simple parts of higher organisms, possess certain characteristics which make them well suited to the study of some aspect of development. These are widely known as developmental "systems."

Systems have been used in investigations of all basic developmental processes. Individual mechanisms contributing to development are understood in some detail as a result. However, the characteristics of these systems means that they are best suited for investigations of a few of the processes of development. Information about different processes, obtained from diverse systems, cannot be easily related. It has been even more difficult to actually perform experiments that directly test these relationships. For this kind of work, systems are required that can be used for a range of developmental processes.

The developing vertebrate limb is one such system that can be used to investigate several processes successfully. The embryonic

limb bud consists of undifferentiated mesoderm surrounded by ectoderm (Figure 1, Appendix I). The limb system has been used for studies of cell division, differentiation, tissue interactions, cell death and morphogenesis. As a result, it can also be used to find out how these processes interact. These processes interact closely during distal outgrowth, when the limb grows out from the flank of the embryo (Figure 2, Appendix I).

Cell division is one of the most obvious processes involved in outgrowth. In the chick embryo limb bud, the mitotic index is slightly lower at the apex than in the proximal core at early stages. From stage 22 to 24 this changes. The mitotic index drops throughout the limb bud at this time, but there is less of a decrease at the apex. This leaves the apex with the highest relative division rate (Janners and Searls, 1970). Further work by Janners and Searls (1970) showed that the drop in mitotic index is caused by an increasing number of cells leaving the division cycle, and not by an increase in cell cycle time.

Formation of proximodistal (flank to limb tip) pattern is also closely associated with outgrowth. Pattern formation, in general, is the spatial organization of differentiated cell types which gives an organism or structure its form (Wolpert, 1978). Specifically, the proximodistal limb pattern requires that a cell in the chick wing not simply differentiate into bone, but together with other cells forms the humerus, radius or ulna, or one of the digits (Figure 3, Appendix I). This close association has been experimentally demonstrated. Fate maps of presumptive limb structures can be drawn

by tracing markers placed in the limb bud at early stages. In birds, such fate mapping experiments show that the early wing bud contains mostly cells that will form proximal structures and that the capacity for forming more distal structures lies with a relatively few cells in the apex of the wing bud. Increasingly complete distal structures are formed as outgrowth proceeds, according to these fate maps (Stark and Searls, 1973; Figure 4, Appendix I). The capacity for further outgrowth is also localized in the apex of the limb bud, and is dependent on apical ectoderm. This ectoderm lies on the distal edge of the limb bud and is thicker than other limb ectoderm. In the wing bud its thickest point is at the distal and posterior edge. At this point it may consist of four or five layers of pseudostratified columnar cells. This thickened distal ectoderm has been termed the apical ectodermal ridge (Figure 1, Appendix I). The removal of the ridge stops further outgrowth (Saunders, 1948; Summerbell, 1974). The removal of ridge ectoderm produces a limb with missing distal structures; the earlier the ridge is removed, the greater is the extent of missing distal structures (Saunders, 1948; Summerbell, 1974; Figure 5, Appendix I). Saunders concluded after this work that there is a "zone of apical growth" in the limb bud that "gradually lays down the tissues for the proximal and then, in succession, the more distal parts of the wing." The zone of apical growth depends on the presence of ectoderm for development. There has been some question whether the ridge promotes limb outgrowth directly or whether other limb ectoderm and mesoderm may act as intermediaries (Amprino, 1965; Amprino and Amprino-Bonetti, 1967). However, overwhelming evidence leaves little

doubt that outgrowth and formation of distal structures are aborted when the ridge is removed (Saunders, 1948; Amprino and Camosso, 1955; Zwilling, 1955; Hampé, 1959; Barasa, 1960; Summerbell, 1974). The ridge itself does not directly specify proximodistal pattern. Rather, the ridge permits outgrowth, and through outgrowth proximodistal pattern can be expressed. This has been shown by transplanting old ridges onto young limb buds. When such transplants are allowed to develop, they do not exhibit proximodistal pattern deficiencies (Rubin and Saunders, 1972).

Current theories dealing with limb outgrowth logically take this association with pattern formation into account. Wolpert, Summerbell and Lewis have proposed that that proximodistal limb pattern arises as a direct result of the outgrowth at the tip of the limb bud. This proposal has generally been called the "progress zone" model (Summerbell, Lewis and Wolpert, 1973; Summerbell and Lewis, 1975; Summerbell, 1977; Wolpert, 1978). Their model is based on two distinctive properties of the limb bud apex: the outgrowth inducing influence of the apical ectodermal ridge and the difference in mitotic rates between subridge and more proximal mesoderm. The model proposes that the dividing tip of the limb bud constitutes a progress zone which is uncommitted in terms of proximodistal pattern. This zone is estimated to extend about 0.30 mm back from the tip of the limb, based on the observation that differentiation does not occur closer than 0.40-0.50 mm from the tip of the limb during outgrowth and that skeletal elements of the three segments are about 0.30 mm long when they first appear. According to the model, the width of the progress

zone remains constant during outgrowth. This constant width means that dividing cells are continually pushed out of the progress zone. The cells that leave the progress zone during early stages of outgrowth end up in the proximal part of the limb. Those cells that leave during later stages will end up in more distal regions. Just how cells are "informed" whether they will become part of proximal or distal structures is one of the model's central concepts. The model proposes that cells measure the time they spend in the progress zone and thereby determine their position in the limb. Cells then form appropriate proximodistal structures based on that information. The more time cells spend in the progress zone, the more distal the structure they should form.

The progress zone model makes several predictions which can be experimentally tested. The salient prediction is that cells from increasingly older progress zones should form increasingly distal structures only. A progress zone from a later limb bud can be grafted in place of a progress zone on a younger limb bud. Such a transplant should generate a limb with missing "middle" level proximodistal structures, since the host stump would contain only the cells for proximal structures already present, and the grafted, older progress zone would have the capacity to develop only more distal structures. The converse operation can also be performed. A progress zone from an early stage limb bud grafted in place of one on an older limb bud should generate a limb with an extra set of proximal structures in the middle of the limb. These extra proximal structures would be contributed by the younger, grafted progress zone. Such predicted

results have been observed when these kinds of experiments are performed on the chick embryo wing bud (Summerbell, Lewis and Wolpert, 1973; Summerbell and Lewis, 1975; Summerbell, 1977; Wolpert, 1978; Figure 6, Appendix I).

How cells measure the time they spend in the progress zone, if in fact they do at all, is as yet unresolved. It has generally been assumed that cells count division cycles as a sort of "clock" mechanism (Wolpert, 1978). Presently there is no direct experimental evidence concerning this. Cells might be able to keep time by an internal clock which is not directly dependent on mitosis, in light of work done on the multinucleate slime mold Physarum (Kauffman, 1977). Cells could also acquire pattern specification in a way that is not related to keeping time.

There is also some reason to believe that cells outside the progress zone may be able to acquire or change pattern specification. Kieny (1977) performed transplants similar to those of Summerbell, Lewis and Wolpert, but used chick and quail limb buds. In "distal to proximal" transplants, a presumptive distal-only limb tip was grafted onto a presumptive proximal-only limb stump. Middle level proximodistal structures should have been missing, according to experimental controls and fate map predictions. Instead, regulation occurred; that is, limbs with a normal, complete proximodistal sequence developed. Middle level limb elements developed, many times arising from both host and donor tissue. In reciprocal "proximal to distal" transplants, a young limb bud was grafted onto a stump containing both presumptive proximal and

distal structures. This operation should have extra proximodistal structures. Again, however, some limbs showed more normal proximodistal sequences after development. Partial regulation was observed in those limbs where extra elements were found (Figure 7, Appendix I).

The discrepancy between the results of the transplant experiments of Summerbell, Lewis and Wolpert and those of Kieny has elicited different explanations. Summerbell (1977) questions the regulation observed by Kieny for two reasons. First, he points out that fate maps may not be sufficiently accurate to predict presumptive structures, so normal sequences may not be a result of regulation at all. Second, Summerbell believes more quantitive work than done by Kieny is required. However, the experiments performed by Kieny made use of meticulous controls, and not just fate maps, and have been repeated several times (Kieny, 1964a,b,c; 1967; Kieny and Pautou, 1976). Cellular markers were also employed to determine whether regulating tissue came from host or donor tissue. Some pattern regulation is also reported by Summerbell (1977), although he prefers to use regulation of limb size as a test of the progress zone model.

The time a cell spends in the progress zone does not appear to be the sole means of determining proximodistal pattern. Even if initial specification of pattern does take place as suggested by the progress zone model, some provision should be made for the changes in specification that are necessary for regulation. There may be a population of subridge cells with special properties, but irreversible determination of proximodistal pattern is probably not one of them.

Several investigators have discovered some special properties of subridge mesoderm. Cairns (1975) and Bell and Ede (1978) cultured tissue explants from chick limb buds. These explants were obtained from different proximodistal levels along the limb bud. Cells from subridge explants were more multipolar in shape, maintained closer contacts with neighboring cells, migrated more slowly in culture, and exhibited cell death at earlier stages than cells from more proximal explants. These differences were most striking between subridge and more proximal explants; differences between proximal explants taken from different levels were less noticeable. Cairns (1975) notes that this subridge zone extends about 0.15 mm proximally into the mesoderm. Newman and Mayne (1974) also cultured chick limb bud explants taken from different proximodistal levels. Proximal cells were able to form cartilage colonies or myotubes. Subridge cells did not. Similar properties have been reported by Bonner and Hauschka (1974). They found that distal mesoderm had the lowest capacity for myogenesis in the limb bud. Kosher, Savage and Chan (1979) have reported that suppression of differentiation in distal chick limb bud tissue is dependent on the apical ectodermal ridge. When cultured, cells from distal explants did not undergo chondrogenesis if the ridge was intact on the explant. Chondrogenesis was observed if the ridge was excised. Cells were also able to differentiate in cultures where the ridge was left intact if they migrated at least 0.40 mm away from the ridge. Similar suppression of distal differentiation has also shown to be dependent on apical ectoderm in the regenerating newt forelimb (Globus, Vethamany-Globus and Lee, 1980).

More recent theories accounting for outgrowth, differentiation and proximodistal pattern formation have been proposed in light of this work. These theories generally propose that some substance diffuses proximally away from the apex of the limb bud. As this substance diffuses away, it would set up a proximodistal gradient in the limb bud. Kosher, Savage and Walker (1981) suggest that a concentration of hyaluronate may be set up to inhibit distal differentiation. This theory is supported by their observation that there is, in fact, a proximodistal gradient of hyaluronate in the chick limb bud with the highest concentration at the distal end. This concentration correlates with the progress of cartilage differentiation in the limb bud. They point out that a likely way for this hyaluronate gradient to be maintained is for the ridge to inhibit differentiation in subridge mesoderm and for the subridge mesoderm to synthesize hyaluronate. Kosher and Savage (1981) found that ridge ectoderm contained higher levels of hyaluronate than nonridge ectoderm, suggesting that hyaluronate itself may be one agent used to maintain the subridge zone. Newman and Frisch (1979) have presented a concentration gradient theory to explain proximodistal pattern formation as well as suppression of differentiation. They propose that a macromolecule similar to fibronectin diffuses away from the interface between the ridge and the mesoderm. This protein is presumed to be necessary in some threshold concentration for precartilage cell interactions and, therefore, differentiation. Diffusion is assumed to occur in the extracellular matrix of undifferentiated cells. A diffusion gradient could form

"standing waves" according to this model. As the limb bud grows, and differentiates at the proximal end, the size of the available "diffusion chamber" would change. Calculations based on actual physical limb bud measurements show that these changes would lead to one, then two, and finally three areas of cartilage formation respectively corresponding to the humerus, radius and ulna, and the three digits of the chick wing. However, this scheme would lead to the immediate chondrogenesis of the distal area of the limb bud. Newman and Frisch therefore conclude that a chondrogenesis-inhibiting substance such as hyaluronate must also diffuse away from the limb bud apex. They further explain that the pattern regulation reported by Kieny (1977) could be caused by a new, continuous diffusion chamber in the host-donor graft limb bud. West, Lanza, Rosenbloom, Lowe and Holtzer (1979) have an alternate theory of proximodistal pattern formation, but it also includes a concentration gradient of fibronectin as a central feature.

The experimental evidence supporting these theories is limited at this time. Melnick, Jaskoll, Brownell, MacDougall, Bessem and Slavkin (1981) find nonrandom patterns of fibronectin in the chick limb bud which might be consistent with the Newman-Frisch model. They point out that fibronectin may be just one of many macromolecules acting in pattern formation, though. The exact function of fibronectin during development is not well characterized presently, either. It is not clear how the Newman-Frisch model accounts for the formation of well developed skeletal elements up to the level of arrested outgrowth after the ridge has been removed, although some

proposals have been advanced. In most of these models, the problem of reversibility of proximodistal pattern has only been peripherally addressed. The evidence concerning inhibition of differentiation is also limited. It is not known when during development the ridge is first necessary for possible inhibition. Nor is it known how gradually cells in subridge mesoderm leave this influence. There is little information as to whether the size of the subridge zone changes during development.

The purpose of the experiments described in the following chapters is to begin to determine when and where in the limb bud controls of differentiation and proximodistal pattern formation act. Attempts to relate outgrowth, differentiation and pattern formation will be impeded until this can be done. Two series of experiments are presented. The first experiments consist of cultures of limb bud tissue explants to more completely map commitment to differentiation in the limb bud. In the second group of experiments, a systematic series of proximodistal transplants test commitment to proximodistal pattern. Pattern regulation is the assay used for this. Myogenesis is the assay used for differentiation.

The chick embryo (Gallus domesticus) has been chosen for use in these experiments. The chick embryo offers many advantages to the developmental biologist and much of the work concerning limb development has been performed on this organism. Fertilized eggs are relatively inexpensive. They may be used at any precise developmental stage after an appropriate period of incubation. Transplants and other operations can be performed on the embryo and the eggs then

reincubated for further development. Such procedures are as yet impossible with mammalian embryos. In addition, chick development is well characterized (Lillie, 1952).

CHAPTER II

DIFFERENTIATION

Introduction

Mesoderm cells of the avian embryo limb bud are relatively undifferentiated at early stages of outgrowth. Within this limb bud mesoderm differentiation is first observed at the proximal end and is observed in successively more distal regions as outgrowth proceeds. Cells within 0.40-0.50 mm of the apical ectodermal ridge remain undifferentiated during outgrowth (Searls, 1965; Searls, Hilfer and Mirow, 1972; Thorogood, 1973). This undifferentiated subridge region also has the highest rate of cell division in the chick limb bud after stage 23 (Janners and Searls, 1970). Surgical removal of the apical ectodermal ridge results in arrested limb outgrowth (Saunders, 1948; Summerbell, 1974; Figure 5, Appendix I) and death of cells in the subridge region (Barasa, 1960; Cairns, 1975; Rowe, Cairns and Fallon, 1982).

These observations have lead to the suggestion that the function of the ridge is to prevent differentiation and death in mesoderm cells directly subjacent to it, thereby promoting outgrowth. This is supported by experimental evidence. Subridge mesoderm cells do not readily differentiate in culture, while cells from more proximal limb bud regions can differentiate when cultured. This has been shown for both myogenesis (Bonner and Hauschka, 1974; Newman and Mayne, 1974) and chondrogenesis (Newman and Mayne, 1974; Kosher, Savage and Chan, 1979). Cells from cultured subridge mesoderm explants are able to

differentiate, though, if they migrate at least 0.40 mm away from the attached ridge, or if mesoderm is cultured in the absence of a ridge (Globus and Vethamany-Globus, 1976; Kosher, Savage and Chan, 1979). Cell death also occurs in subridge mesoderm more quickly if no ridge is present than if the ridge is left intact or if a ridge is present in the culture but not in contact with the mesoderm (Cairns, 1975). Other special properties of subridge mesoderm have been observed. When explants taken from different proximodistal levels of the limb bud are cultured, subridge cells are morphlogically distinguishable from those of more proximal explants. Subridge cells are more multipolar in shape, maintain closer contact with neighboring cells, migrate more slowly in culture, and exhibit cell death at a slightly earlier time (Cairns, 1975; Bell and Ede, 1978). Concentrations of hyaluronate are also higher in subridge than more proximal mesoderm (Kosher, Savage and Walker, 1981).

At this point it is not clear how far into the limb bud the subridge zone extends. The region of the limb bud where cells are not able to differentiate when cultured has been reported as probably extending 0.40-0.50 mm proximally from the ridge or simply as the "distal half" of the limb bud. However, the zone of subridge cells with the special properties observed by Cairns (1975) extends only about 0.15 mm proximally. Rowe, Cairns and Fallon (1982) report that cell death is seen in only the distal 0.15-0.20 mm of the limb bud when the ridge is removed. It is also not clear whether the properties of subridge mesoderm change during the course of development.

The purpose of the experiments reported here is to better define where in the limb, and when during the course of development, a special zone of subridge cells is maintained. Specifically, these experiments test how far away from the ridge cells in the limb must be before they are first able to differentiate in culture and how this changes during outgrowth. Myogenesis, as indicated by the formation of myotubes in culture, is used as an assay of ability to differentiate. We also test to what extent the ridge is required to maintain cells in an undifferentiated state.

Materials and Methods

Fertile White Leghorn chicken eggs were obtained from the Poultry Farm of the Department of Animal Science at The University of Tennessee, Knoxville. Prior to incubation eggs were stored for up to a week at 10° C. Incubation was carried out in a standard unit providing the necessary humidity and egg rotation at a temperature of 37-38° C. After three days of incubation the eggs were prepared for use by first drilling a hole in the narrow end of the egg, through which 2-3 ml albumin was drawn out by a sterile syringe. This lowered the embryo from the shell of the egg. The drilled hole was then resealed with melted paraffin. Square windows were then cut in the shell of each egg, just over the embryo, by using a hand held electric drill with a cutting wheel attachment. The square was removed and discarded and the window was covered with Parafilm (American Can Company) and sealed with an electrically heated iron. Eggs were then reincubated for an appropriate period of time without rotation. Embryos were staged according to the staging series of Hamburger and Hamilton (1951; Figure 2, Appendix I).

Limb tissue explants were removed from right and left wing buds of stage 17-23 embryos. Two explants were taken from each wing bud: an "apical" or subridge explant, and a "proximal" explant which was subjacent to the apical explant. Both explants were about 0.10-0.20 mm thick and about 0.50-0.70 mm long. Explants were taken from the region under the thickest part of the apical ectodermal ridge at all stages (Figure 8, Appendix I). Camera lucida drawings were made of the limb bud after each explant was removed in order to record the position of each explant in the limb bud. The ridge, and dorsoventral ectoderm, were normally left intact on the explants. However, at stage 19 and 22 the procedures were repeated with the exception that the ridge was first surgically removed immediately prior to explant removal. The dorsoventral ectoderm was not disturbed in ridge removal explants. All operations were performed with microscalpels made from fine tungsten wire, brought to a point in a high propane flame. Scalpels were cleaned between each use by passing through a low alcohol flame.

Explants were temporarily held in sterile petri dishes filled with cold phosphate buffered saline until they were ready to be placed in culture. Covered plastic microtest plates with rows of separate culture wells were used for myogenesis assay explants. Wells which were to contain explants were filled with 12 ul of sterile medium made up from 8.5 ml minimal essential medium, 0.7 ml horse serum, 0.3 ml fetal calf serum and 0.5 ml 9-day chick embryo extract. All other wells were filled with sterile distilled water to help maintain humidity. Explants were then placed individually in the culture

wells with the distal end of the explant facing up. Tissue was cultured in a CO_2-water incubator at 37-38°C. Culture medium was not changed during the course of incubation.

Cultures were inspected every 24 hours through an inverted phase microscope. Explants that did not adhere to the bottom of the culture well, or became contaminated, were excluded from further observation or analysis. The presence or absence of myotubes was recorded each day. Cultures were observed carefully for four days in order to determine if they were able to form myotubes. They were watched for up to fourteen days to make sure that results obtained in the first four days accurately reflected whether or not the explants could form myotubes.

Results

After one day in culture, cells spread out from the explant across the bottom of the culture well. Cell density appeared to be the lowest at the outer edges of the mass of spread cells. Some cell death was seen on the first day, as demonstrated by debris and macrophages. This may have been partly caused by the excision of the explant. Myotubes were generally first observed in proximal explants on day two or three of incubation. Usually myotubes first appeared along the periphery of the spread cells. One day after the first myotubes were seen, the culture became crowded with myotubes. Further myogenesis was not noticed after this. These myotubes were present for the remainder of the culture period. Cultures that did not exhibit myogenesis by four days of incubation still did not even when cultured for up to ten more days, in our experience. Extensive

macrophages became apparent in the center of the explants by three or four days. Cultures that did not undergo myogenesis were seen to recede back towards the explant by day four. No evidence of chondrogenesis was encountered in any culture well.

Table 1 (Appendix II) shows when myotubes were first observed in cultures of apical and proximal explants taken from the several stages of wing buds. At every stage, the majority of apical cultures never contained myotubes (Figure 9, Appendix I). At stage 19, with the ridge removed, 65% of the apical cultures showed no myogenesis at all. From 80-100% of the apical cultures did not undergo myogenesis at all other stages. The majority of proximal cultures contained myotubes by the second or third day of incubation (Figure 9, Appendix I). A minimum of 70% of proximal cultures contained myotubes by day three at every stage. At most stages 85-95% of the proximal cultures were myogenic by the third day. Very clearly, cells in the apical cultures generally do not form myotubes and the cells from proximal cultures generally do. This trend is evident, but to a less marked degree, for cultures of explants taken from wing buds younger than stage 20. There is a slight increase in myotube formation in apical cultures, and a slight decrease in myogenesis in proximal cultures, at these early stages. Removal of the apical ectodermal ridge still resulted in no myogenesis in 95% of the apical cultures at stage 22 and in 65% of the apical cultures at stage 19.

Examination of camera lucida drawings of limb buds generally confirmed that the thickness of each explant was about 0.10-0.20 mm thick. Most were about 0.15 mm thick. None exceeded about 0.20 mm.

These measurements are approximate since three camera lucida drawings of each limb bud were required to record the position of the two explants: one before any explant is removed, and one after the removal of each explant. Movement of the embryo in the egg and other inherent errors contribute to a certain inaccuracy in these measurements.

Discussion

One of the goals of these experiments was to determine how far proximally in the limb bud cells must be before they can differentiate in culture. Camera lucida drawings of apical and proximal explants indicate that they were about 0.10–0.20 mm thick. From these measurements alone, though, it is difficult to tell just how far into the wing bud cells must be before they can differentiate in culture. In cultures of proximal explants, it may be that only cells from the very proximal edge of the tissue participate in myogenesis. If this is the case, cells could be prevented from differentiating in culture when they are taken from limb bud sites as far back from the apex as 0.40 mm. However, there is some reason to think that this suppression is most complete back to only about 0.15–0.20 mm from the apex (Gay and MacCabe, 1979). A distance of 0.15 mm also corresponds to the width of the subridge zone described by Cairns (1975), and 0.20 mm is the estimate of the width of the strip of cell death that appears along the apex when the ridge is removed (Rowe, Cairns and Fallon, 1982). It is less than the 0.40–0.50 mm extent of inhibition observed by Kosher, Savage and Chan (1979) and less that the 0.30 mm "progress zone" proposed by Summerbell, Lewis and Wolpert (1973).

Recent work has demonstrated a requirement for somite cells to migrate into the limb bud before myogenesis can occur (Chevallier, Kieny and Mauger, 1976; 1977; 1978; Chevallier, Kieny, Mauger and Sengal, 1977; Christ, Jacob and Jacob, 1974; 1977). A possible reason for the lack of myogenesis is that premuscle somite cells may not have migrated into the limb bud any closer than 0.20 mm from the apex at these stages. McLachlan and Hornbruch (1979), however, have shown that wing bud mesoderm can form muscle in the absence of somite cells. Our results provide some evidence for a specialized zone subjacent to the ridge in either case. If the lack of myogenesis in subridge explant cultures was caused by an absence of premuscle somite cells, it might be because some "barrier" was preventing their migration into this region. Cairns (1975) and Bell and Ede (1978) have reported that cells from this subridge zone maintain closer cell contacts in culture and may have different adhesive strengths than more proximal cells. If, in fact, somite cells have not invaded this region, these or other properties may be acting to set up a barrier against them. Myogenesis may not be the only type of differentiation that cannot take place in subridge mesoderm cultures. There is good evidence that chondrogenesis also does not occur in subridge cultures, but can in more proximal tissues in culture (Newman and Mayne, 1974; Kosher, Savage and Chan, 1979). Whether the achondrogenic region coincides with our amyogenic region has not been determined. Estimates of the width of the achondrogenic region by Kosher, Savage and Chan are wider than our estimate of the amyogenic apical zone, but they used different culture conditions.

Several important results are summarized in Table 1 (Appendix II). The first is that myogenesis is generally prevented in apical cultures, and generally has taken place by the second or third day in proximal cultures, for all tested stages. This trend is less defined before stage 20 but is still present. Ridge removal has no real effect on the ability to form myotubes at stage 22 in either apical or proximal cultures, and little if any real effect at stage 19. If there is a partitioning of premyogenic and nonmyogenic zones, this partitioning seems to be incomplete before stage 20. At these early stages, perhaps the mesoderm is being programmed by the ridge to set up a partition between premyogenic and nonmyogenic zones. Consequently, when the ridge is removed at stage 19, some apical explants are able to form myotubes in culture because their programming is not yet complete. After stage 20, and certainly by stage 22, apical zone cells may be so irreversibly programmed that they do not undergo myogenesis in culture even after the ridge has been removed. It may be that apical cells must slowly move away from the zone, as they normally would in the limb bud during outgrowth, in order to lose this programming and so become able to differentiate. We suggest that there may be many gradations of commitment to differentiation throughout the limb bud. At the very distal tip of the limb bud, cells are not able to differentiate at all; even in culture, and with the ridge removed, cells do not undergo myogenesis. Somewhat more proximally, cells have still not begun to differentiate in the limb bud itself, but may be able to under appropriate culture conditions. Even more proximal cells actually

differentiate in the limb bud. Specification of proximodistal pattern may also take place in a similar manner, with cells at the tip of the limb bud being almost totally uncommitted, and more proximal cells becoming more and more committed to pattern formation. The apical zone characterized by our results and those of others may be analogous to the progress zone proposed by Summerbell, Lewis and Wolpert (1973). The progress zone may be closer to 0.15-0.20 mm wide than 0.30 mm wide if this is true. We have tested this hypothesis and will report the results in an upcoming paper.

CHAPTER III

PATTERN FORMATION

Introduction

Skeletal elements of vertebrate limbs become arranged in a precise proximodistal sequence during development (Figure 3, Appendix I). How is this proximodistal pattern formed in the limb? One explanation is that limb structures are formed in a proximal to distal sequence during limb bud outgrowth (Saunders, 1948; Figure 5, Appendix I). This view is represented in a detailed manner in the "progress zone" model proposed by Summerbell, Lewis and Wolpert (Summerbell, Lewis and Wolpert, 1973; Summerbell and Lewis, 1975; Summerbell, 1977; Wolpert, 1978). This model states that cells become committed to form a particular part of the proximodistal pattern based on the time they spend in the growing tip, the progress zone, of the limb bud. Thus pattern should already be determined in tissues proximal to the progress zone. An alternate view is that pattern is not irreversibly determined in proximal limb bud tissues, at least at early stages, and so the limb tip is not exclusively responsible for the specification of proximodistal pattern (Kieny, 1977).

Proximodistal transplant experiments have been used to test whether proximal limb tissues are irreversibly committed to a specific component of pattern. Such transplant experiments create conditions that test the regulative ability of the limb bud by imposing presumptive abnormalities in the proximodistal sequence. If a limb bud with an experimentally imposed, presumptively abnormal sequence

develops into a limb with a more normal proximodistal sequence, then the limb bud has regulated to correct these abnormalities. This indicates that proximodistal pattern was not irreversibly determined at the time of the transplant operation. Two general kinds of transplant experiments are used. In "proximal to distal" level transplants, limb bud grafts are produced that carry extra presumptive proximodistal segments. Missing presumptive segments are created in "distal to proximal" level transplants.

Support for the views of Summerbell, Lewis and Wolpert, and of Kieny, can be obtained from the results of transplant experiments. Summerbell, Lewis and Wolpert have reported that the chick wing bud generally does not regulate when limb bud tips presumably including complete progress zones, are transplanted to different proximodistal levels (Figure 6, Appendix I). Exceptions may be observed if transplants are made within the region of the progress zone itself (Summerbell and Lewis, 1975) or at early stages of outgrowth (Summerbell, 1977). These results have been cited as evidence that responsibility for proximodistal pattern formation resides in the progress zone, and not in more proximal limb bud regions where the host and donor are grafted together. However, results from similar experiments, as exemplified by the work of Kieny, show that the avian limb bud can regulate when presumptive abnormalites are imposed on the proximodistal sequence (Hampé, 1959; Kieny, 1964a,b,c; 1967; Kieny and Pautou, 1976; Kieny, 1977; Figure 7, Appendix I).

These apparent discrepancies have hindered attempts to formulate a more comprehensive understanding of proximodistal limb pattern formation. In fact, the question of how pattern is set up has been overshadowed to some extent by the controversy of whether or not the chick limb bud can regulate along its proximodistal axis. These apparent discrepancies may be explained in part by the numerous differences in experimental procedures, and in the methods of analysis, used by the different groups of investigators. We believe that any attempt to increase the understanding of proximodistal pattern formation requires a knowledge of what regions of the limb bud can regulate and how this changes during the course of development. This knowledge is difficult to obtain solely from previous results.

The experiments reported here test the ability of the chick limb bud to undergo proximodistal pattern regulation over a range of conditions, yet with experimental variables kept to a practical minimum. The procedures employed in these experiments avoid introducing extra variables by mixing tissues from different stages or from different species within a transplant. Amputation and grafting methods are as uniform as practical. Very large presumptive abnormalities are created by graft operations so that any possible regulation will be more easily identified. Leg tissue is transplanted onto wing tissue in all operations to further help in identifying any structures affected by regulation. Finally, transplants are performed at both stage 21 and 23. This is done to determine how regulative ability changes in the limb bud during development. We present some conclusions about proximodistal limb

pattern formation based on comparisons of the procedures and results of our experiments and those of others.

Materials and Methods

Fertile eggs from White Leghorn Chickens were supplied by the Poultry Farm of the Department of Animal Science at The University of Tennessee, Knoxville. Eggs were stored at 10°C for not more than a week before beginning incubation. Eggs were then placed in an incubator providing the necessary humidity and egg rotation at a temperature of 37-38° C. After three days of incubation a hole was drilled in the narrow end of the egg and 2-3 ml albumin was drawn out through the drilled hole by a sterile syringe. The hole was resealed with melted paraffin. Square windows were then cut in the shell of each egg just over the embryo. A hand held electric drill with a small cutting wheel was used for this. After removing the cut square, the window was covered with Parafilm (American Can Company) and sealed with an electrically heated iron. Eggs were then incubated for a further appropriate period without rotation. Embryos were staged according to the staging series of Hamburger and Hamilton (1951; Figure 2, Appendix I) and transplant operations were performed. Instruments were wiped with 70% ethanol and allowed to dry before each use. Grafted tissues were held in place with thin tungsten wire tacks which were also sterilized before each use. Two tacks were used for each graft: one placed near the anterior edge of the graft limb bud and one near the posterior edge. Camera lucida drawings were made of all limb buds after operating. Parafilm was used to reseal the egg windows.

Eggs were placed back in an incubator after the operations. They were not rotated during postoperative incubation. One day after the operation, grafts were inspected. Graft limb buds showing internal bleeding or incomplete healing were discarded. Tungsten wire tacks were removed from successful transplants at this time. Seven or eight days after the operations limbs were cut from the embryos and fixed in formalin. Limbs were stained for cartilage elements with Victoria Blue B as recommended by Bryant and Iten (1974) and cleared with methyl salicylate. Limbs were analyzed by identifying the stained skeletal structures in each (Figure 3, Appendix I).

Several sets of transplant operations were performed. All types of transplants were done at both stage 21 and 23. In all cases donor leg bud tissue was transplanted onto host wing bud tissue. All operations were performed on right side limb buds. Three groups of limbs were analyzed. "Control host" wings developed from wing buds that were amputated at the same level as the host wing buds used for grafts, but did not have donor tissue grafted on. These were used to estimate at what level along the proximodistal axis of the host wing bud amputations were made. "Donor" legs developed from leg buds that had donor tissue removed. These allowed an estimate of where the amputation was made in the proximodistal axis of the donor leg bud. "Graft" limbs resulted from transplants of donor leg bud tissue onto host wing bud tissue. Different embryos were used for each "control host," "donor" and "graft" operation. Proximal to distal level transplants and distal to proximal level transplants were carried out as described (Figures 10 and 11, Appendix I).

Proximal to distal. Proximal to distal level transplants create graft limb buds with more presumptive proximodistal structures than normal. Host wing buds were amputated through the junction between the presumptive zeugopodium (radius and ulna) and presumptive autopodium (digits). Donor leg tissue was obtained by amputation through the presumptive stylopodium (femur). Some exceptions were made for stage 23 transplants. A few of these donor leg buds were amputated through the level of the presumptive zeugopodium (tibia and fibula) to determine if the level of amputation of the donor would affect the results. At both stages, proximal to distal transplants were intended to produce grafts containing a presumptive wing stylopodium and zeugopodium with a presumptive leg zeugopodium and autopodium attached distally. Elements of host autopodium and donor stylopodium may also be included. This means that most proximal to distal transplants should have had at least one extra zeugopodium.

Distal to proximal. Distal to proximal level transplants create graft limb buds with fewer than normal presumptive proximodistal segments. At each stage, two kinds of host wing bud amputations were employed. "Flank" hosts were amputated through the junction between the presumptive shoulder and the presumptive stylopodium (humerus). Amputations for "humerus" hosts were through the level of the presumptive stylopodium. Donor leg buds were amputated through the level of the junction of the presumptive zeugopodium (tibia and fibula) and presumptive autopodium (ankle and toes). Donor tissue was cut out using a tungsten wire scalpel so that a strip of a uniform width of 0.10–0.20 mm could be obtained. "Flank" grafts were designed to produce graft limb buds containing a presumptive wing shoulder with

a presumptive leg autopodium lying distally. "Humerus" grafts were different in that a portion of wing stylopodium was intended to be included.

A further distal to proximal level transplant group was done to test whether the age of donor tissue might be a factor affecting results. This was done by transplanting a donor leg bud presumptive autopodium from a stage 23 embryo onto a stage 21 host wing bud amputated through the presumptive stylopodium. Except for the mixed stages, these operations were performed in the same manner as the other distal to proximal transplants.

Results

In some cases transplants failed to heal properly or graft embryos died before their limbs could be analyzed, especially in the case of the proximal to distal transplants. For this reason results were analyzed in two ways. When possible, the skeletal structures of donor legs were directly compared to the graft limbs where they had been transplanted. However, because this was not always possible, resulting structures from donor legs and from graft limbs were also pooled for each stage and operation. Results are presented as the percentage of limbs possessing a specific skeletal composition for each operational type, at each stage.

Proximal to distal. The results of the proximal to distal transplants are summarized in Table 2 (Appendix II). At both stage 21 and 23, control host wings (those that did not receive a graft) contained at least a radius and ulna, indicating that the control

hosts were, in fact, amputated near the junction of the zeugopodium and autopodium. In many cases elements of posterior digits were also found. This can be predicted by comparing the plane of amputation of the control host wing buds (Figures 10 and 11, Appendix I) to the fate map of the wing bud (Stark and Searls, 1973; Figure 4, Appendix I). In several cases the radius and ulna angled apart from each other (Figure 12, Appendix I).

All stage 21 donor legs consisted of a portion of the femur (Figure 12, Appendix I). Sixty-nine percent of the stage 23 donor legs also contained only femur or part femur (Figure 12, Appendix I). The remainder of the stage 23 donor legs contained more distal structures. These were from embryos where the amputation was deliberately made at a more distal level. Of these, the majority had no structures distal to the middle level of the tibia and fibula.

Proximal to distal graft limbs had skeletal structures that were largely predicted by the analysis of control host wing and donor leg skeletal structures. In every case, organization consisted of wing structures in the proximal part of the limb and leg structures in the distal part. In no cases were graft limbs observed that had only wing structures or only leg structures (Figure 13, Appendix I). Of all proximal to distal grafts, only one exhibited a normal, three-segmented proximodistal sequence. This was found in a stage 21 graft limb. A host wing humerus, and radius and ulna, had a donor leg distal half tibia joined to the radius, with ankle and toes distal to that. Eighty percent of the stage 21 grafts and 59% of the stage 23 grafts contained skeletal elements of at least two extra

proximodistal segments. Forty-two percent of the stage 23 graft limbs had one extra proximodistal segment. Most of these had tissue from donor leg buds that were amputated distal to the femur. Of stage 21 graft limbs, there were five cases where the same donor and graft embryos survived. None contained the correct number of proximodistal segments. Ten donor-graft pairs survived in the stage 23 group. Again, no correct three-segment proximodistal sequences were observed. For both stage groups of surviving donor-graft pairs, the skeletal elements of the donor leg and the leg structures of the graft limb were complementary. That is, if the donor leg consisted of half a femur, the leg structures of the graft consisted of a portion of femur, tibia and fibula, and ankle and toes. Two exceptions were observed in the stage 21 donor-graft pair group. In these cases, a portion of femur was observed in the donor legs, but no femur was seen in the graft limbs.

The skeletal elements of the graft limbs were arranged in several configurations. Sometimes the donor leg tibia and fibula were inserted between the host wing radius and ulna, or alongside of them, so that the host and donor zeugopodia were aligned. In some cases the radius and ulna joined the tibia and fibula at a right angle. There were also several cases of a sequential arrangement, with distal host wing elements adjoining proximal donor leg elements. Some donor zeugopodia contained only a tibia, or a tibia with an incomplete fibula. (See Figure 13, Appendix I, for these configurations).

Distal to proximal. Results from the distal to proximal transplants are presented in Table 3 (Appendix II). Attempts were made to amputate the host wing bud at two levels for both stages; through the shoulder-humerus junction ("flank" group), and through the humerus ("humerus" group) (Figure 14, Appendix I). This attempt was not very successful for the stage 21 control host wings. While 83% of the "flank" group control hosts did not contain any humerus, 72% of the "humerus" group control hosts also did not contain humerus. This operation was a greater success when performed at stage 23. Eighty-two percent of the stage 23 "flank" control hosts had no humerus. The "humerus" group, though, had limbs containing about half of a humerus in 71% of the cases. The difference between the stage 21 groups and the stage 23 groups can probably be explained by the fact that the distance between the presumptive shoulder and the presumptive middle humerus is small at stage 21 (Stark and Searls, 1973; Figure 4, Appendix I). At stage 23 this distance is greater, making it easier to consistently make amputations at the two different levels. Host amputations for stage 23 to stage 21 transplants were done in the same way as the stage 21 "humerus" hosts, so seperate control hosts were not analyzed.

Donor legs contained a femur and most of the tibia and fibula in all donor leg groups (Figure 14, Appendix I). Only two donor legs from all groups did not contain elements distal to the middle of the tibia and fibula. All other donor legs had a proximodistal sequence terminating between the distal quarter of the zeugopodium and the proximal quarter of the autopodium.

Graft limbs from all distal to proximal groups had wing structures in the proximal end of the limb and leg structures in the distal end. Never were all wing or all leg graft limbs observed (Figure 15, Appendix I). Results for distal to proximal graft limbs are described by group. Representative graft limbs are displayed in Figure 15 (Appendix I).

Stage 21 "Flank." Twenty-five percent of the stage 21 "flank" grafts had skeletal structures predicted by the host wing and donor leg controls. In these cases a foot was attached to a shoulder. Forty-four percent of the graft limbs contained what appeared to be a complete humerus between the shoulder and foot. In 25% of the "flank" grafts, the humerus branched out into a "Y"-shaped structure at the distal end. These have been grouped in the "correct sequence" class in Table 3 (Appendix II) because they appear to be attempts to form a radius and ulna. This hypothesis is supported by the results from the experimental groups described below. There was only one case of a correct sequence with a zeugopodium composed of leg elements. This was judged to have probably been transplanted with donor tissue, based on a comparison of the camera lucida drawing of the donor leg bud with fate maps. Thirteen pairs of donor-graft embryos from this group survived together. Five contained no humerus or less than half a humerus between the shoulder and foot. Nine contained a whole humerus or a humerus branching out into a "Y."

Stage 21 "Humerus." Fifteen percent of the stage 21 "humerus" group graft limbs had structures predicted by control host wing and donor leg analyses: a host shoulder with a donor ankle and toes

immediately adjacent. Limbs with a part or whole humerus were observed in 30% of the cases. Almost half of the "humerus" grafts, 46%, had some zeugopodial elements. These appeared to be wing structures. Of these, one limb had a humerus branching into a "Y." All others appeared to be more like a normal radius and ulna. There was a single case of leg zeugopodial elements. Again, this probably was transplanted with donor tissue. Seven pairs of donor-graft embryos survived. One had no humerus, four had half or a complete humerus, and three had structures believed to be a radius and ulna.

Stage 23 "Flank." No zeugopodial structures were seen in the stage 23 "flank" group graft limbs. Structures predicted by analysis of host and donor control limbs were observed 28% of the time, with no stylopodium or zeugopodium present. Wing stylopodial structures were seen in 72% of the graft limbs. Of these, 50% possessed a complete humerus. The distal end of one such humerus was curved sharply toward the adjacent ankle and toes. There were seventeen surviving donor-graft pairs. Nine contained either no humerus, or less than half a humerus, and eight had a complete, or nearly complete, humerus.

Stage 23 "Humerus." There was a single case of a graft limb with structures predicted by controls. Sixty-one percent of the stage 23 "humerus" group graft limbs had most of a humerus present. A further 21% had a humerus with a "Y" branching from the distal end. Analysis of the surviving donor-host pairs supports this data. Of seven pairs, one had no humerus, five had a complete or nearly complete humerus, and one had a humerus-"Y" structure.

Stage 23 to Stage 21 "Humerus." At minimum, a complete humerus was present in all graft limbs of this group, in addition to the predicted shoulder and foot elements. Eighty-seven percent of these graft limbs also had wing zeugopodial structures. Four of these cases were a humerus branching into a "Y;" the rest consisted of a nearly complete or complete radius and ulna. Seven of the eight donor-graft pairs showed wing zeugopodial pairs between the predicted host and donor elements.

Discussion

The experiments described here were specifically designed to find out where in the limb bud, and at what stages of development, cells become irreversibly committed to forming a specific part of proximodistal pattern. For the purposes of our experiments, "proximodistal pattern" is the sequence made up of the stylopodium, zeugopodium and autopodium. "Regulation" means that appropriate segments must be added or removed in order to restore a presumptively abnormal sequence to this three-segment configuation. Regulation of limb size, or rearrangement of abnormal sequences after pattern has already been determined, may be important aspects of limb development but are not useful assays of pattern commitment.

Significant regulation does not appear to have taken place in our proximal to distal transplants, according to our definition of regulation. Except for a single graft limb, extra segments are observed in grafts from both stages. The skeletal structures found in proximal to distal graft limbs agree closely with what an analysis of control host and donor limbs predicts. Donor legs consisted of

part or all of the femur, except for those leg buds deliberately amputated at a more distal level, suggesting that a small part of the presumptive femur may have been transplanted along with the presumptive tibia, fibula and foot in donor tissue. Typical control host wings contained humerus, radius and ulna, and some digit elements. Graft limbs generally consisted of humerus, radius and ulna, digits, tibia and fibula, and foot.

The possibilty of a very low level of regulation is not ruled out by our results. No femur elements were observed in stage 21 graft limbs. It could be that the proximal portion of the donor tissue can regulate to a limited extent by "erasing" the presumptive femur. However, the lack of a femur in the graft limbs could also be caused by a possible small amount of necrosis around the amputation plane of the donor leg bud. This would lead to a decrease in the amount of femur in a given donor leg or graft limb. Amputation of donor leg buds is probably a traumatic operation (Figures 10 and 11, Appendix I) and so some tissue death might be expected. It is important to remember, however, that our proximal to distal transplants were designed to avoid the problems of trying to assess such low levels of regulation. At least one extra presumptive proximodistal segment, and most often several, were created by the grafting procedures. These extra presumptive segments were not removed by regulation.

Some form of secondary regulation, acting after pattern is irreversibly determined, may be operating. In our results and those of Kieny (1977), homologous proximodistal segments sometimes

aligned. This occasionally produced a graft limb sequence of host stylopodium, host and donor zeugopodium together, and donor autopodium. Evidence that this is actually a mechanism of secondary regulation is not strong. Alignment of homologous proximodistal segments occurred infrequently in our results. Homologous segments aligned at right angles, or end-to-end, were as common as side-by-side configurations. These are probably all the results of uneven healing between host and donor tissue. Control host wings often showed a distal splitting apart of radius and ulna, and donor tissue appeared to heal into such a "split" center of host tissue one day after the transplant was performed. Similar healing effects were observed in distal to proximal transplants one day after the operation. In other cases, when host and donor zeugopodia were aligned, the donor fibula was often absent or only partially complete. Similar results have been reported by Kieny (1977). It is not clear that this meets the definition of proximodistal regulation. The complete tibia was present in all graft limbs. Probably the loss of some of the fibula represents a form of anteroposterior regulation. It may be difficult for the limb bud to accomodate four areas of chondrogenesis, side-by-side, in one segment.

The results of our distal to proximal transplants provide a striking contrast. The data demonstrate that many graft limbs have a proximodistal sequence that is more normal than that presumably created by the grafting operation. Several graft limbs exhibited normal sequences. Control host wings contained some or none of the humerus. Donor legs contained femur, tibia and fibula. This

suggests that a presumptive foot and perhaps the distal end of the presumptive tibia and fibula were transplanted onto the proximal part of the humerus, or just onto the shoulder. The extra humerus, or humerus branching into a "Y" structure, or humerus, radius and ulna that were found in some graft limbs are good candidates for products of regulation. Considering the size of the gap in the presumptive proximodistal sequence produced by the transplant procedures, there are often more wing structures than can be accounted for on the basis of tissue death around the host wing bud amputation plane. Such large discrepancies did not show up in comparisons of donor legs and graft limbs in proximal to distal transplants, and there an analogous operation was used. The abnormal appearance of some of these elements, such as a humerus branching into a "Y," also suggests regulation. Humerus-"Y" structures were never observed in any contol host limbs or in proximal to distal graft limbs.

Wing, or wing-like, skeletal elements were observed between host and donor elements in distal to proximal graft limbs. Whether these structures arose from host wing tissue, donor leg tissue, or both is not known with certainty because cellular markers were not used. The possibility exists that these wing structures originated from cells of the donor leg bud. Fallon (personal communication) has evidence that leg mesoderm can give rise to wing structures under the following conditions: it must be transplanted to a wing bud so that it is not in contact with the apical ectodermal ridge, and presumptive leg cells must become "integrated" with cells of the wing mesoderm. In our opinion, the observed wing structures were more likely to have arisen

from host wing tissues. There are some observations that may indirectly support this view. Saunders, Gasseling and Cairns (1959) have reported that when leg mesoderm is transplanted to a wing bud, it can give rise to leg structures, even if it is not in contact with the ridge. In our proximal to distal graft limbs, clearly identifiable leg and wing structures exist side-by-side when homologous segments are aligned. Leg mesoderm did not form wing structures as far as can be observed. The conditions of our experiments, and those of Saunders, Gasseling and Cairns, probably did not produce the required integration of leg and wing cells necessary for development of leg mesoderm into wing structures.

Careful examination of the results of distal to proximal transplant experiments reveals two general trends. First, the ability of host tissue to participate in the regulation of proximodistal pattern declines with the age of the host tissue and also in more proximal tissue in host limb buds of a given age. (In our experiments, only host wing tissue appeared to fill in presumptive deficiencies by regulation). Second, the host-donor junction in the graft limb bud must be sufficiently close to the apex of the graft bud before regulation can occur. We would like to suggest some proposals concerning proximodistal pattern formation based on these observations. We suggest that the first beginnings of pattern specification take place in a narrow region at the tip of the limb bud. This "zone" is probably no more than 0.20 mm wide, but may be even narrower. Cells in this zone are very labile, are not able to differentiate, and are morphologically different from cells of more

proximal limb bud regions (Newman and Mayne, 1974; Cairns, 1975; Bell and Ede, 1978; Gay and MacCabe, 1979; Kosher, Savage and Chan, 1979; Gay and MacCabe, unpublished). Cells immediately proximal to this zone are very weakly committed to forming a specific part of limb proximodistal pattern. As cells become more proximal in the limb bud as a result of outgrowth, this commitment becomes stronger. Finally, when they are far enough away from the apical zone, or have been away from it long enough, their commitment becomes irreversible. This increase in level of commitment could be accomplished in two ways. Individual cells might pass through several stages of increasing commitment. An alternate way would be that commitment is an all or nothing event, so that an increasing proportion of cells would become committed at more proximal levels.

These hypotheses can explain the different results of proximodistal transplants performed by ourselves and others. By examining how different results are produced by different procedures, the way proximodistal pattern is controlled during development can be better illustrated. The simplest experiment that is addressed by these hypotheses is removal of the apical ectodermal ridge. Removal of the ridge leads to arrested outgrowth and a distally incomplete proximodistal pattern. More complete patterns are produced by delayed ridge removal (Saunders, 1948; Summerbell, 1974; Figure 5, Appendix I). Rowe, Cairns and Fallon (1982) have demonstrated that mesoderm cells die in a subridge area extending to 0.20 mm into the limb bud following ridge removal. This means that ridge removal probably ceases outgrowth by effectively killing the apical zone. The

skeletal structures present in the developed chick wing after ridge removal correspond closely with the presumptive structures in the fate maps of Stark and Searls (1973) that are proximal to the proposed 0.10-0.20 mm apical zone (Figure 4, Appendix I).

A uniform, narrow strip of donor leg tissue was grafted onto host wing tissue at stage 21 and 23 in our distal to proximal experiments. A greater degree of regulation was observed if the operation was performed at stage 21 than at stage 23. Also, the more distally the host wing bud was amputated, the more complete was the observed regulation. The stage 23 donor-stage 21 host transplants demonstrate that the differences in regulation at the two stages are caused by the changing ability of the host stump to participate in regulation, and not by a changes in the donor tissue. These mixed stage transplants produced at least as much regulation as the stage 21 distal to proximal transplants. (Actually, even more regulation was observed. This is probably because a stage 23 donor tip fits the host stump better than a stage 21 tip. Analogous findings have been reported by Kieny, 1977). Thus, the ability of the stump to participate in regulation decreases proximally with age, suggesting a wave of proximal to distal commitment through the limb bud as outgrowth proceeds.

Our results indicate that cells from the host wing tissue probably were the major contributors to skeletal elements produced by regulation. This means that for regulation of presumptive deficiencies to occur, two steps are required. First, uncommitted cells would have to be brought back under the influence of the apical

zone so that they could divide. Second, either they would have to receive new instructions as to what part of the pattern they should form, or they must act on previous pattern instructions. An insufficient number of uncommitted host stump cells would lead to incomplete regulation.

Kieny (1977) also observed regulation in distal to proximal transplants. In one procedure, stage 22 donor chick tissue was grafted onto stage 20-21 host quail tissue. Quail tissue was used as a cellular marker, since it can be distinguished from chick tissue (Le Dourain and Barq, 1969). Elements produced by regulation had contributions from both host and donor cells. This could be explained by the fact that Kieny's donor limb bud tips were cut straight across, rather than of a uniform width following the profile of the limb bud as in our experiments. Straight amputations produce a very narrow area of donor tissue at the anterior and posterior corners. Cells from the host stump could then have been brought more completely into the apical zone than in our experiments. Actual mixing of donor and host tissue in a new apical zone might lead to intercalated elements that are produced by both. In another procedure, the segment of host tissue was reversed on its proximodistal axis prior to grafting on a donor limb tip. No regulation occurred as a result of this procedure. According to our hypotheses, this might be because the host tissue adjacent to the donor piece was too "proximal" to participate in regulation. The proximal end of stage 20 or 21 quail limb buds may be too committed for regulation.

Summerbell, Lewis and Wolpert (1973) and Summerbell and Lewis (1975) performed distal to proximal transplants by grafting the distal quarter of a stage 24 wing bud onto a stage 19 host wing stump. Such grafts did not regulate. This cannot be explained by the inability of the host wing tissue to participate; at stage 19, it should have been able to very well. Instead, lack of regulation was probably caused by transplanting a relatively wide donor tip. Their procedure created a graft limb bud where the host tissue was more than 0.30 mm away from the apex of the graft. This is too far away, according to our estimate of the range of apical zone influence. Summerbell (1977) also removed slices from the middle of stage 19-25 chick wing buds and grafted distal tips back onto the stumps. Slices of various widths were taken from limb bud regions corresponding to the presumptive elbow. Regulation, including humerus-"Y" structures, was observed at early stages. From stage 22 on, significant regulation was not observed. The lack of regulation in later stages can be explained by the procedures employed. At early stages, such as stage 19, sufficiently uncommitted host tissue is adjacent to a fairly narrow tip. At stage 24, however, the grafted tip is too wide for regulation when narrow slices are removed, and host tissue adjacent to the graft junction is not distal enough to be sufficiently uncommitted when wide slices are removed.

The proximal to distal transplants reported by Summerbell, Lewis and Wolpert (1973), Summerbell and Lewis (1975), and in this report, did not regulate by removing extra presumptive proximodistal segments. The distal tip of the host tissue should have been

uncommitted enough to do so in our experiments. Failure to regulate in both sets of experiments was probably caused by the use of wide donor limb bud tips. In all experiments, these tips exceeded 0.30 mm from apex to host tissue. Regulation did occur when Kieny (1977) transplanted the tip of a stage 19-20 limb bud onto a stage 22 host bud. These grafted donor tips were narrower. In many cases, such graft limb buds regulated by "erasing" the distal parts of host wing structures. This is what our hypotheses would predict, if uncommitted tissue at the tip of the host part of the graft limb bud is brought under the influence of a transplanted apical zone. It is hard to see how "erasing" presumptive structures could be consistent with pattern specification based on the number of division cycles spent in the apical zone, as proposed in the progress zone model. Such an erasing would seem to require cells to go through reverse division cycles.

The results of mesoderm reaggregation experiments (MacCabe, Saunders and Pickett, 1973) provide an interesting application for our hypotheses. In these experiments, mesoderm from stage 17-22 limb buds was dissociated, reaggregated, and inserted back into limb ectoderm. After allowing reaggregated limb buds to develop on the flanks of chick embryos, skeletal elements were analyzed. Distal elements seemed to be the most normal. More proximal elements did not show as normal a proximodistal configuration. We interpret this as being caused by a mixing of committed and uncommitted cells during reaggregation, so that there was no complete pattern commitment in any region of the reaggregated bud. However, cells near the apical

ectodermal ridge could become part of a new apical zone. Here uncommitted cells could be instructed as to what part of the proximodistal pattern they will form. Proximal regions would have little commitment and might be too far away from the apical zone to receive new commitment.

These hypotheses we have suggested share some similarities with the progress zone model of Summerbell, Lewis and Wolpert (1973). There are also some basic differences. Our proposed apical zone is narrower than the progress zone. Commitment of cells to a specific pattern role after they leave the limb bud apex is gradual, in our view. The relative levels of commitment can be measured by the relative abilities of cells to participate in regulation. The progress zone model specifies that pattern is set up as a result of the amount of time cells spend in the specialized apex of the limb bud. We think that interactions between the apical ectodermal ridge and subridge mesoderm, and between cells of subridge mesoderm, are probably responsible for assigning pattern specification. Actual commitment to this specification, and commitment to the process of differentiation itself, may require many other interactions. It is likely that there may be many graded zones of different kinds of interactions throughout the limb bud as a result.

LIST OF REFERENCES

LIST OF REFERENCES

AMPRINO, R. (1965). Aspects of limb morphogenesis in the chicken. In Organogenesis. (ed. R. DeHaan and H. Ursprung), pp. 225-281. New York: Holt, Rinehart and Winston.

AMPRINO, R. & AMPRINO-BONETTI, D. (1967). Experimental observations on the development of ectoderm-free mesoderm of the limb bud in chick embryos. Nature, London 214, 826-827.

AMPRINO, R. & CAMOSSO, M. (1955). Recerche spermentali sulla morfogenesi delgi arti nel pollo. J. exp. Zool. 129, 453-493.

AMPRINO, R. & CAMOSSO, M. (1959). On the role of the "apical ridge" in the development of the chick embryo limb bud. Acta Anat. 38, 280-288.

BARASA, A. (1960). Conseguenze dell'ablazione della cresta ectodermica apicale sullo svilluppo dell'ablozzo dell'ala nell'embrione di pollo. Riv. Biol. 52, 257-292.

BELL, D. & EDE, D. (1978). Regional differences in the morphology and motility of mesodermal cells from the early wing-bud of normal and talpid mutant chick embryos. J. Embryol. exp. Morph. 48, 185-203.

BONNER, P. & HAUSCHKA, S. (1974). Clonal analysis of vertebrate morphogenesis. I. Early developmental events in the chick limb. Devl. Biol. 37, 317-328.

BRYANT, S. V. & ITEN, L. E. (1974). The regulative ability of the limb regeneration blastema of Notophthalmus viridescens: experiments in situ. W. Roux' Arch. 174, 90-101.

CAIRNS, J. M. (1975). The function of the ectodermal apical ridge and distinctive characteristics of adjacent distal mesoderm in the avian wing bud. J. Embryol. exp. Morph. 34, 571-583.

CHEVALLIER, A., KIENY, M. & MAUGER, A. (1976). Sur l'origine de la musclature de l'aile chez les oiseaux. C. r. hebd. Séanc. Acad. Sci., Paris D 282, 309-311.

CHEVALLIER, A., KIENY, M. & MAUGER, A. (1977). Limb-somite relationship: origin of the wing musculature. J. Embryol. exp. Morph. 41, 245-258.

CHEVALLIER, A., KIENY, M. & MAUGER, A. (1978). Limb-somite relationship: effect of removal of somitic mesoderm on the wing musculature. J. Embryol. exp. Morph. 43, 263-278.

CHEVALLIER, A., KIENY, M. MAUGER, A. & SENGAL, P. (1977). Developmental fate of somitic mesoderm in the chick embryo. In Vertebrate Limb and Somite Morphogenesis. (ed. D. Ede, J. Hinchliffe and M. Balls), pp. 421-432. Cambridge University Press.

CHRIST, B., JACOB, H. J. & JACOB, M. (1974). Uber den Ursprung der Flugelmuskulatur. Experimentelle Untersuchungen mit Watchel und Huhnerembyonen. Experientia 30, 1446-1448.

CHRIST, B., JACOB, H. J. & JACOB, M. (1977). Experimental analysis of the origin of the wing musculature in avian embryos. Anat. Embryol. 150, 171-186.

GAY, S. & MACCABE, J. (1979). The ectodermal ridge and myogenic potential in the developing limb. Symp. Amer. Soc. Zool., Tampa 1979, Amer. Zool. 19, 925.

GLOBUS, M. & VETHAMANY-GLOBUS, S. (1976). An in vitro analog of early chick limb bud outgrowth. Differentiation 6, 91-96.

GLOBUS, M., VETHAMANY-GLOBUS, S. & LEE, Y. C. I. (1980). Effect of apical ectodermal cap on mitotic cycle and cartilage differentiation in regeneration blastemata in the newt, Notophthalmus viridescens. Devl. Biol. 75, 358-372.

HAMBURGER, V. & HAMILTON, H. (1951). A series of normal stages in the development of the chick embryo. J. Morph. 88, 49-92.

HAMPÉ, A. (1959). Contribution a l'etude du developpement et de la regulation des deficiencies et des excedents dans la patte de l'embryon de poulet. Arch. Anat. Micro. Morph. exp. 48, 345-478.

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

KAUFFMAN, S. (1977). Dynamic models of the mitotic cycle: evidence for a limit cycle oscillator. In Mathematical Models for Biological Discovery. (ed. S. Levin), pp. 225-281.

KIENY, M. (1964a). Régulation des excédents et des déficiencies du burgeon d'aile de l'embryon de poulet. Arch. Anat. Morph. exp. 53, 29-44.

KIENY, M. (1964b). Étude du méchanisme de la régulation dans le développement du burgeon de membre de le embryon de poulet. I. Régulation des excédents. Devl. Biol. 9, 197-229.

KIENY, M. (1964c). Étude du méchanisme de la régulation dans le développement du burgeon de membre de le embryon de poulet. II. Régulation des déficiencies dans les chimeras 'aile-patte' et 'patte-aile.' J. Embryol. exp. Morph. 12, 357-371.

KIENY, M. (1967). Phénomènes de régulation de l'ébauche de membre chez l'embryon de poulet. Anat. exp. Morph. 39, 1-37.

KIENY, M. (1977). Proximodistal pattern formation in avian limb development. In Vertebrate Limb and Somite Morphogenesis. (ed. D. Ede, J. Hinchliffe and M. Balls), pp. 87-103. Cambridge University Press.

KIENY, M. & PAUTOU, M. P. (1976). Régulation des excédents dans le développement du burgeon de membre de l'embryon d'oiseau. Analyse expérimentale de combinaisons xénoplastiques caille/poulet. W. Roux' Arch. 179, 327-338.

KOSHER, R. A. & SAVAGE, M. P. (1981). Glycosaminoglycan synthesis by the apical ectodermal ridge of the chick limb bud. Nature, London 291, 231-232.

KOSHER, R. A., SAVAGE, M. P. & CHAN, S. (1979). In vitro studies on the morphogenesis and differentiation of the mesoderm subjacent to the apical ectodermal ridge of the embryonic chick limb-bud. J. Embryol. exp. Morph. 50, 75-97.

KOSHER, R. A., SAVAGE, M. P. & WALKER, K. H. (1981). A gradation of hyaluronate accumulation along the proximodistal axis of the chick limb bud. J. Embryol. exp. Morph. 63, 85-98.

LE DOURAIN, N. & BARQ, G. (1969). Sur l'utilisation des cellules de la caille japonaise comme 'marqueurs biologiques' en embryologie expérimentale. C. r. hebd. Séanc. Acad. Sci., Paris D 269, 1543-1546.

LILLIE, F. (1952). Development of the Chick. (rev. H. Hamilton). New York: Holt, Rinehart and Winston.

MACCABE, J. A., SAUNDERS, J. W. & PICKETT, M. (1973). The control of the anteroposterior and dorsoventral axes in embryonic chick limbs constructed of dissociated and reaggregated limb-bud mesoderm. Devl.Biol. 31, 323-335.

MCLACHLAN, J. C. & HORNBRUCH, A. (1979). Muscle-forming potential of the non-somitic cells of the early avian limb bud. J. Embryol. exp. Morph. 54, 209-217.

MELNICK, M., JASKOLL, T., BROWNELL, A. G., MACDOUGALL, M., BESSAM, C. & SLAVKIN, H. C. (1981). Spatiotemporal patterns of fibronectin distribution during embryonic development. I. Chick limbs. J. Embryol. exp. Morph. 63, 193-206.

NEWMAN, S. & FRISCH, H. (1979). Dynamics of skeletal pattern formation in the developing chick limb. Science 205, 662-668.

NEWMAN, S. & MAYNE, R. (1974). Axial differences in the process of chondrogenesis in the embryonic limb bud. J. Cell Biol. 63, 245a.

ROWE, D., CAIRNS, J. & FALLON, J. (1982). Spatial and temporal patterns of cell death in limb bud mesoderm after apical ectodermal ridge removal. Submitted for publication.

RUBIN, L. & SAUNDERS, J. W. (1972). Ectodermal-mesodermal interactions in the growth of limb buds of the chick embryo: constancy and temporal limits of the ectodermal induction. Devl. Biol. 28, 94-112.

SAUNDERS, J. W. (1948). The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. J. exp. Zool. 108, 363-402.

SAUNDERS, J. W., GASSELING, M. T. & CAIRNS, J. M. (1959). The differentiation of prospective thigh mesoderm grafted beneath the apical ectodermal ridge of the wing bud in the chick embryo. Devl. Biol. 1, 281-301.

SEARLS, R. L. (1965). An autoradiographic study of the uptake of S-35 sulfate during the differentiation of limb bud cartilage. Devl. Biol. 11, 155-168.

SEARLS, R. L., HILFER, S. R. & MIROW, S. (1972). An ultrastructural study of early chondrogenesis in the chick wing bud. Devl. Biol. 28, 127-137.

STARK, R. A. & SEARLS, R. L. (1973). A description of chick wing development and a model of limb morphogenesis. Devl. Biol. 38, 133-151.

SUMMERBELL, D. (1974). A quantitative analysis of the effect of excision of the AER from the chick limb-bud. J. Embryol. exp. Morph. 32, 651-660.

SUMMERBELL, D. (1977). Regulation of deficiencies along the proximo-distal axis of the chick wing-bud: a quantitative analysis. J. Embryol. exp. Morph. 41, 137-159.

SUMMERBELL, D. & LEWIS, J. H. (1975). Time, place and positional value in the chick limb bud. J. Embryol. exp. Morph. 33, 621-643.

SUMMERBELL, D., LEWIS, J. H. & WOLPERT, L. (1973). Positional information in chick limb morphogenesis. Nature, London 224, 492-496.

THOROGOOD, P. (1973). The appearance of muscle protein and myofibrils within the embryonic chick limb bud. J. Embryol. exp. Morph. 30, 673-679.

WEST, C. M., LANZA, R., ROSENBLOOM, J., LOWE, M. & HOLTZER, H. (1979). Fibronectin alters the phenotypic properties of cultured chick embryo chondroblasts. Cell 17, 491-501.

WOLPERT, L. (1978). Pattern formation in biological development. Sci. Amer. 239, 154-164.

ZWILLING, E. (1955). Ectoderm-mesoderm relationship in the development of the chick embryo limb bud. J. exp. Zool. 128, 423-441.

APPENDIXES

APPENDIX I

FIGURES

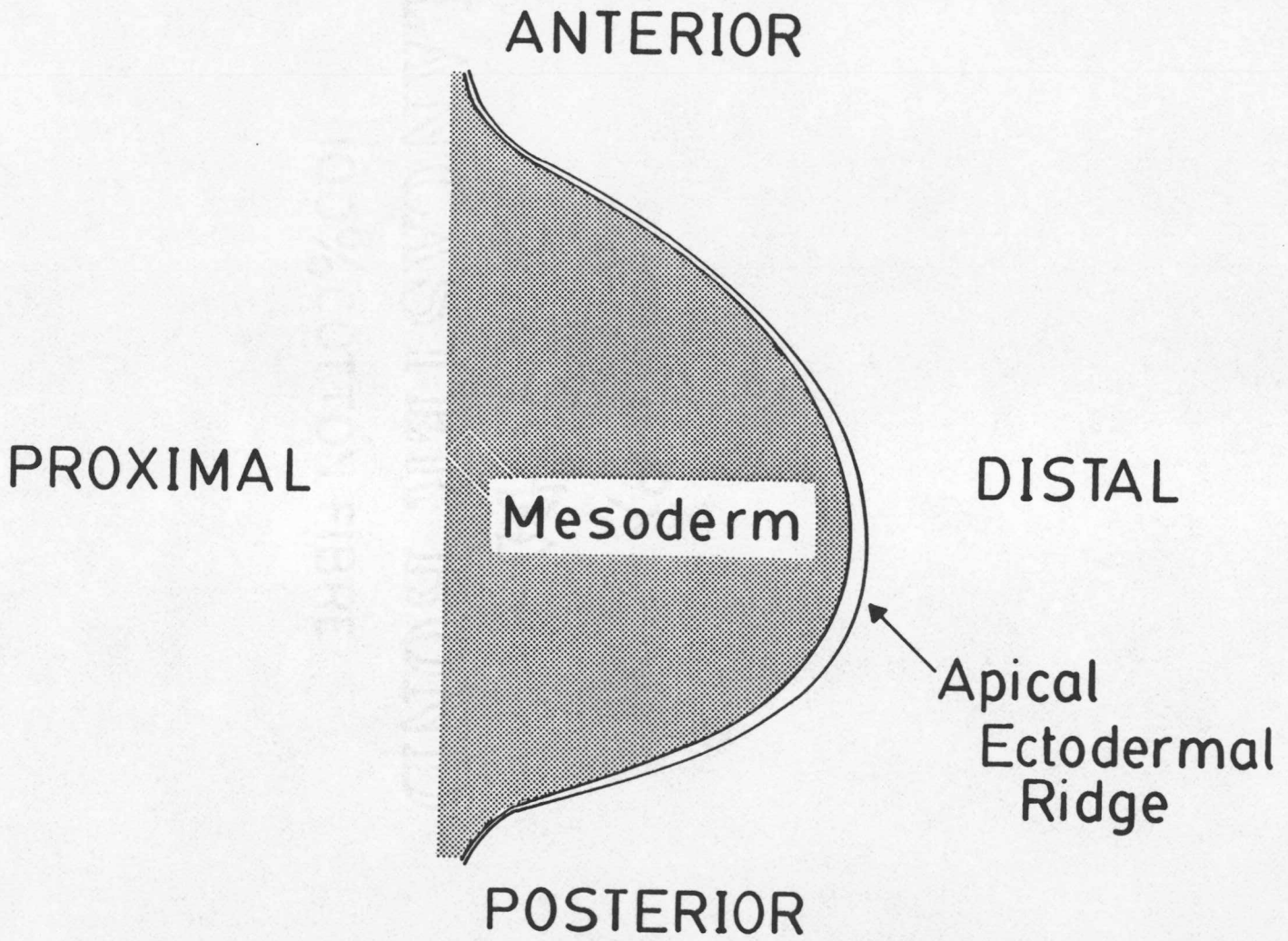


Figure 1. Schematic dorsal view of a stage 21 chick wing bud.

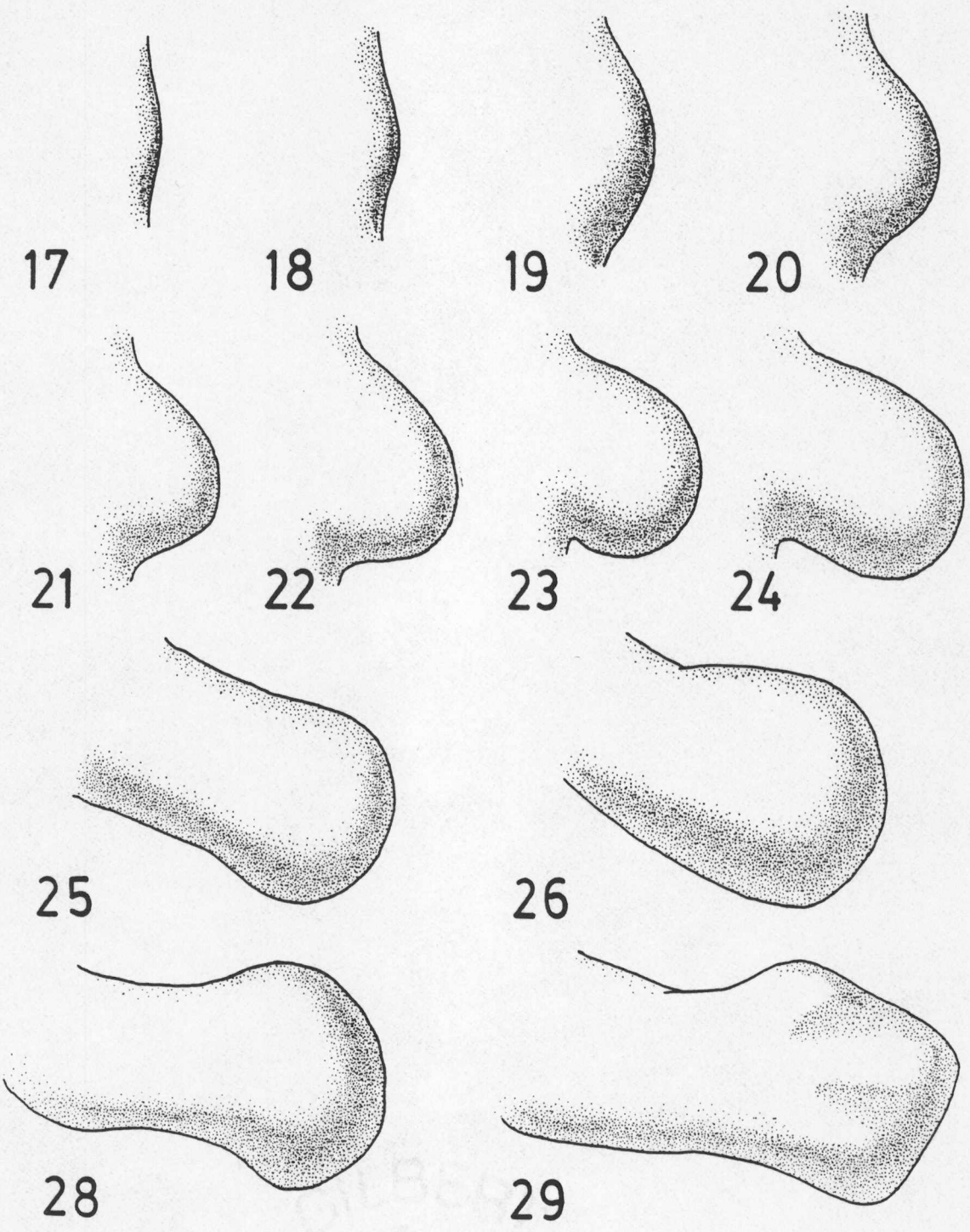


Figure 2. Profiles of chick wing buds during distal outgrowth.

Numbers indicate the staging series proposed by Hamburger and Hamilton (1951).

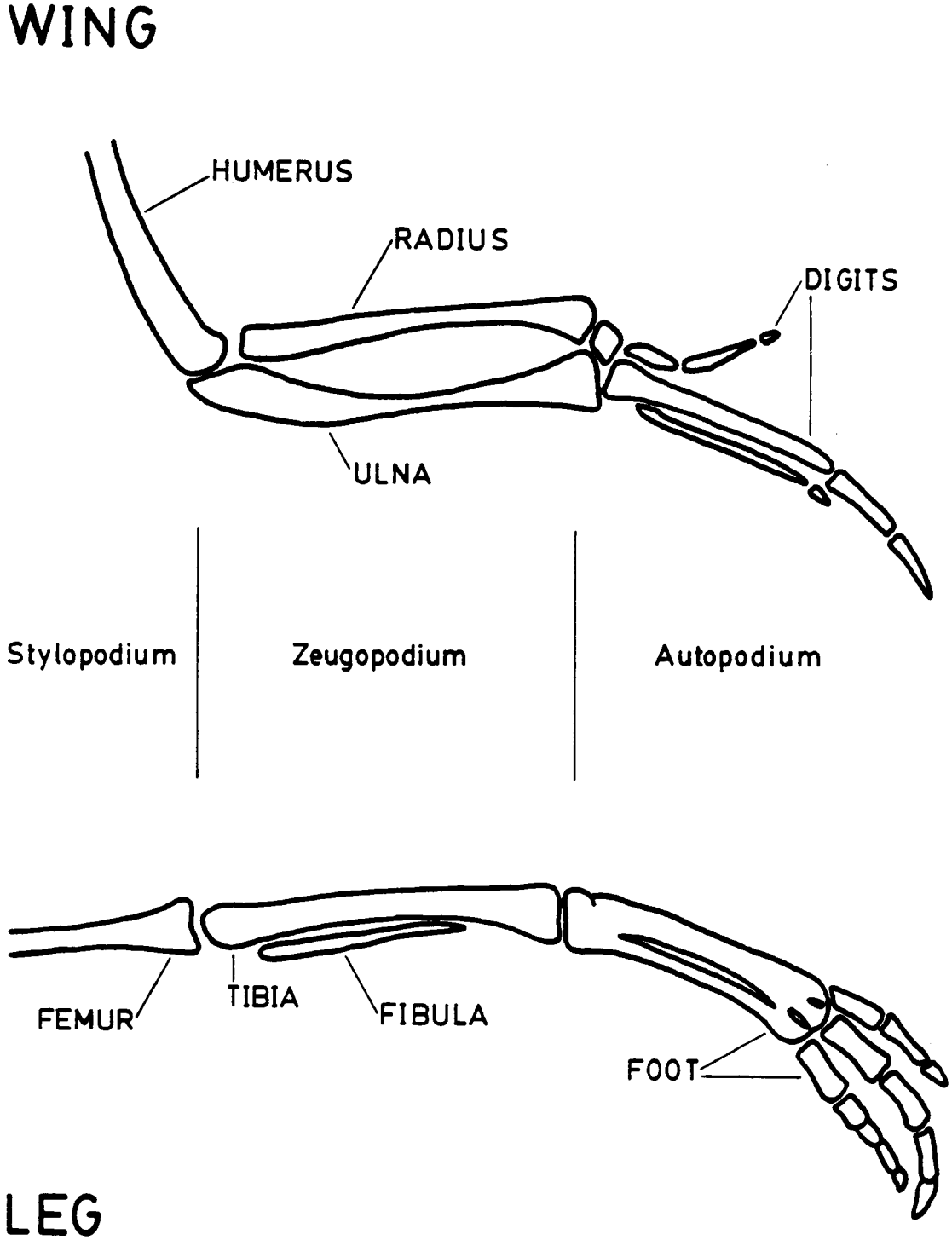


Figure 3. Chicken limb skeletal structures, showing homologous wing and leg proximodistal segments.

Figure 4. Fate maps of chick wing buds at various developmental stages, produced by autoradiographic labelling.

From Stark and Searls (1973).

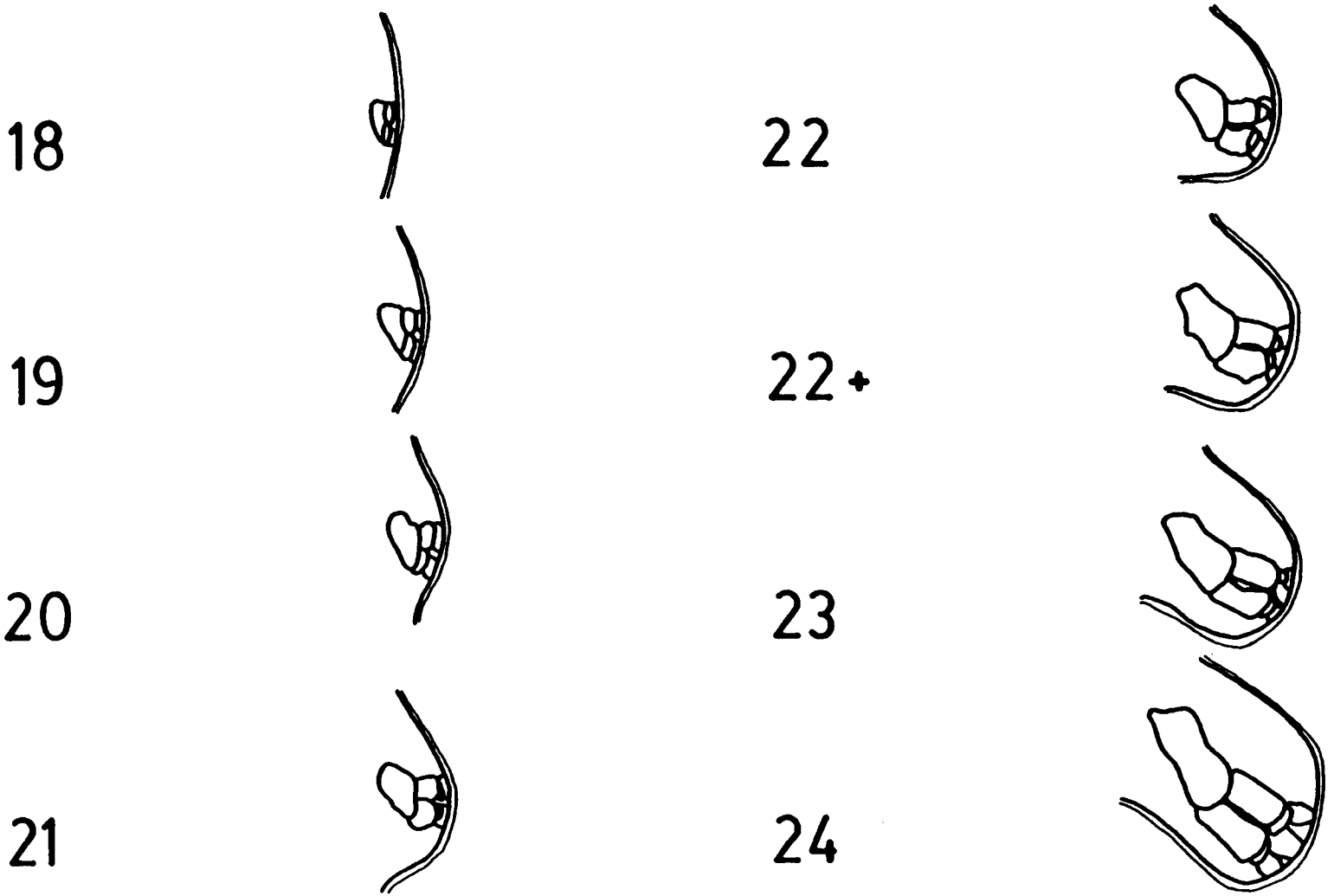


Figure 4.

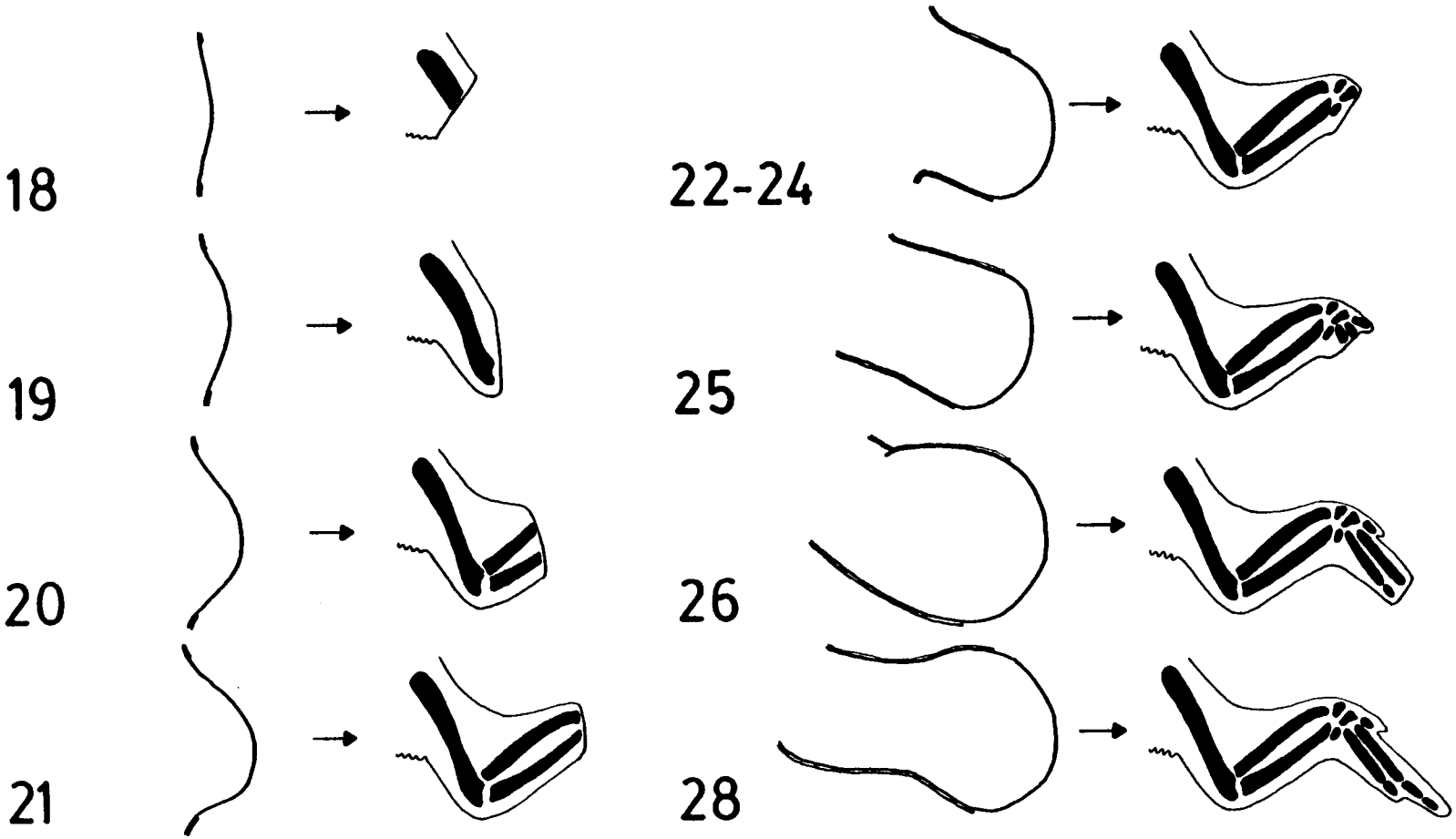


Figure 5. Arrest of outgrowth in chick wing buds produced by excision of the apical ectodermal ridge at various developmental stages.

From Saunders (1948) and Summerbell (1974).

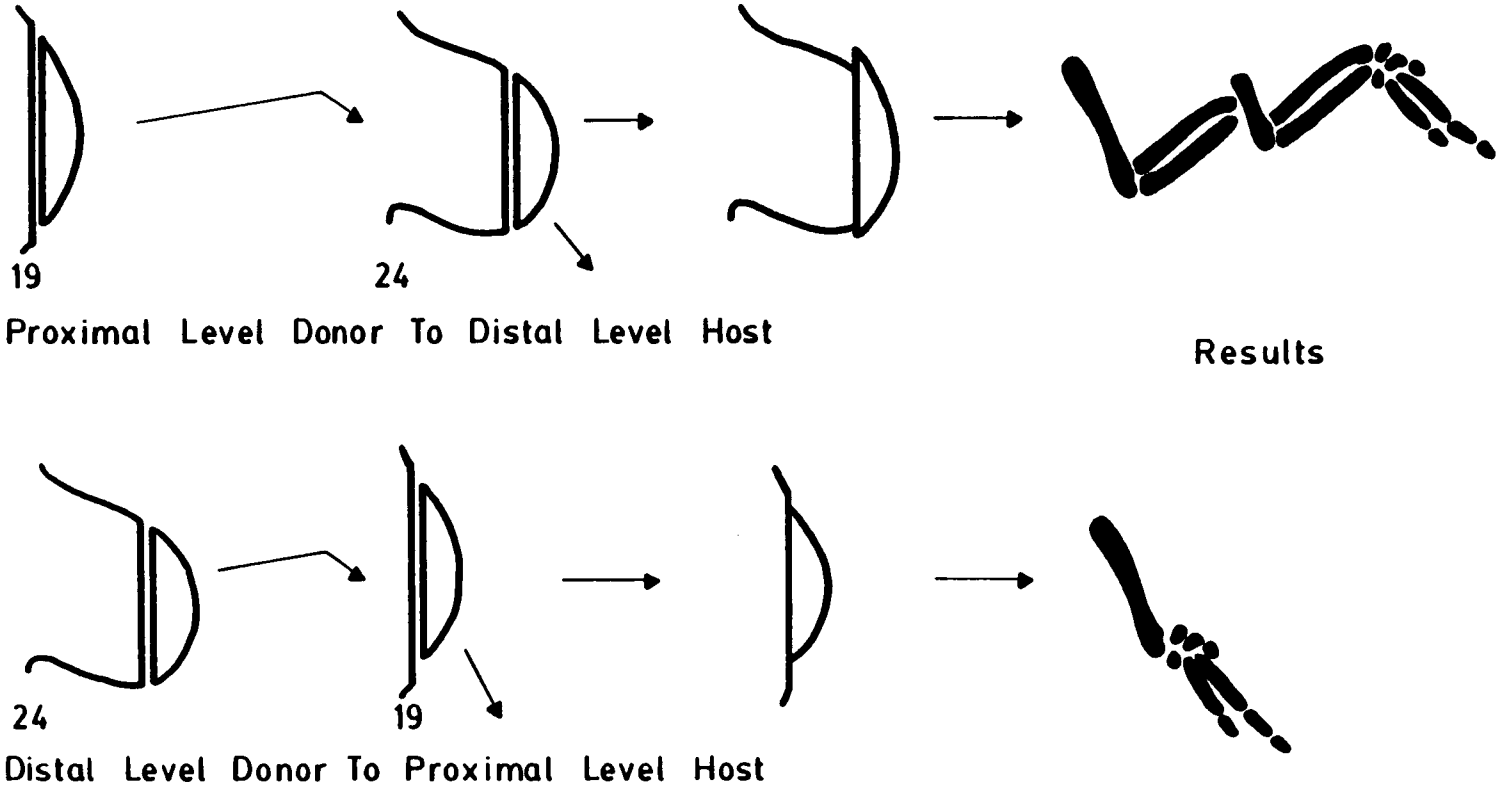


Figure 6. Proximodistal transplants as performed by Summerbell, Lewis and Wolpert (1973).

Numbers below limb buds indicate stages of development at transplant. Typical resulting skeletal structures are shown.

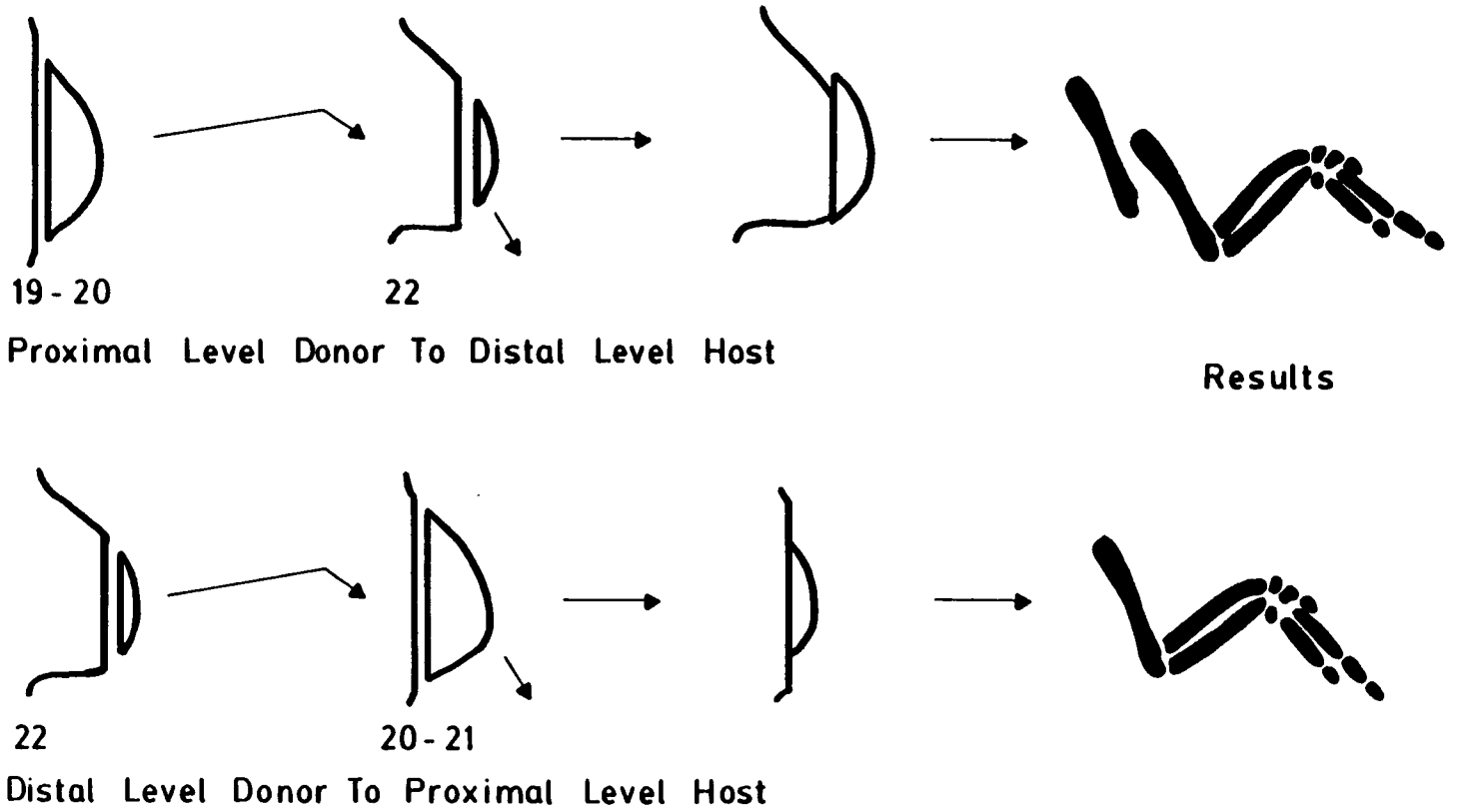


Figure 7. Proximodistal transplants as performed by Kieny (1977).

Numbers below limb buds indicate stages of development at transplant. Typical resulting skeletal structures are shown.

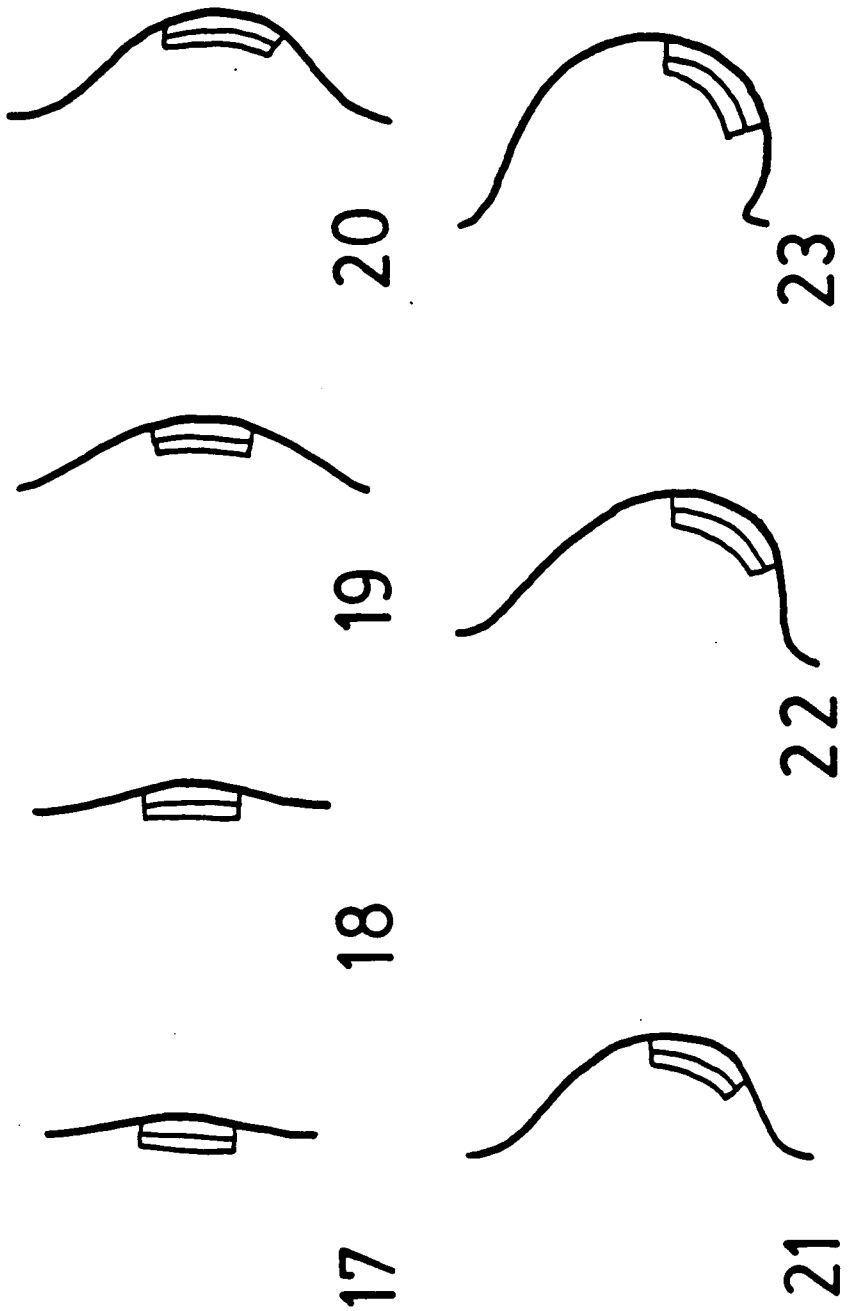


Figure 8. Apical and proximal explants removed from chick wing buds for myogenesis assay.

Numbers indicate stages of development.

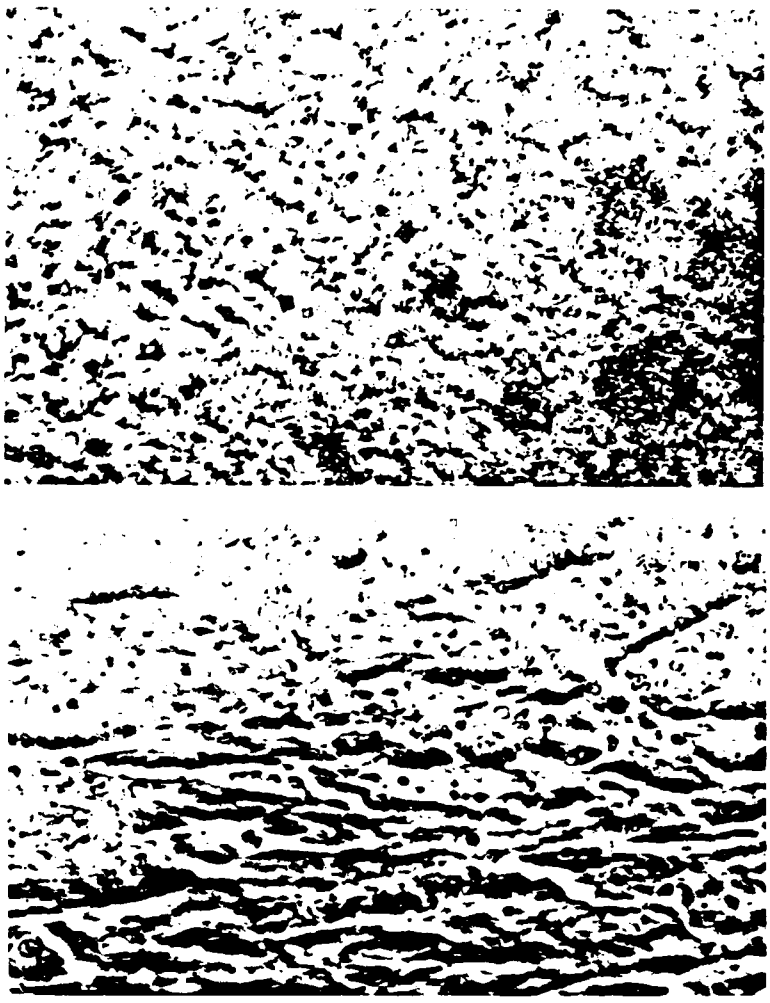


Figure 9. Inverted phase contrast photomicrographs of cells from apical and proximal explants after 72 hours incubation.

Magnification: 480x. (a.) Apical culture. Cells are undifferentiated. Dark, large macrophages indicate cell death. (b). Proximal culture. Myotubes are the dominant cell form, indicating differentiation.

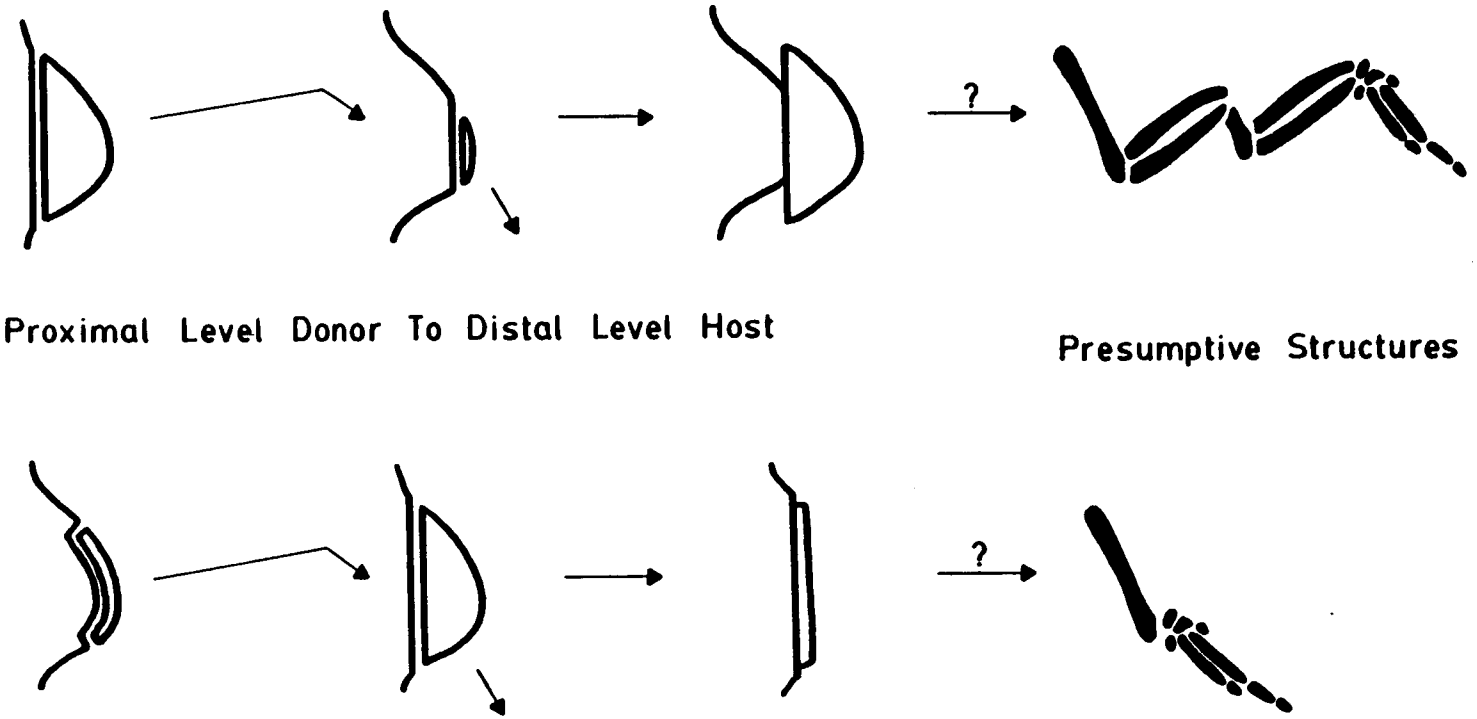


Figure 10. Proximodistal transplants as reported in Materials and Methods, Chapter III, for stage 21 chick limb buds.

Presumptive structures are shown in terms of wing elements; however, leg tissue was transplanted onto wing tissue.

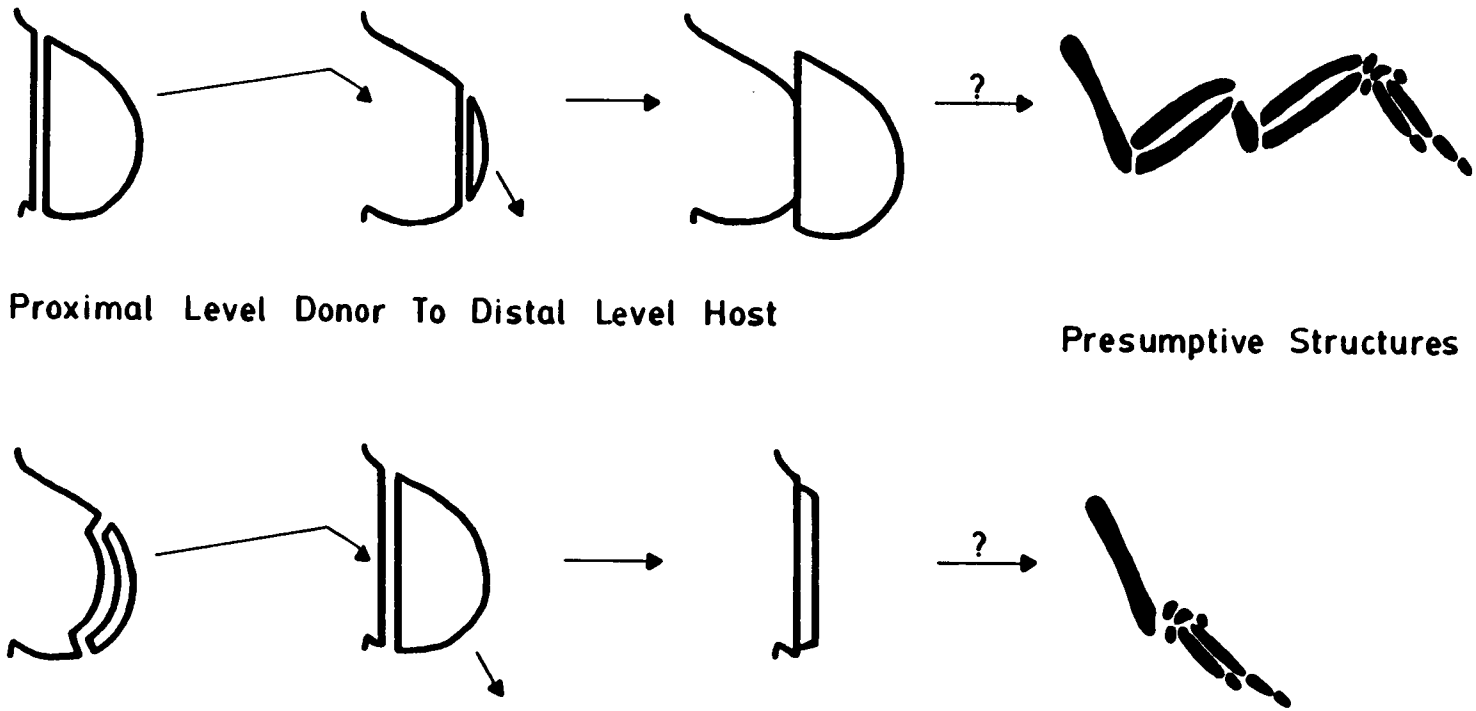


Figure 11. Proximodistal transplants as reported in Materials and Methods, Chapter III, for stage 23 chick limb buds.

Presumptive structures are shown in terms of wing elements; however, leg tissue was transplanted onto wing tissue.

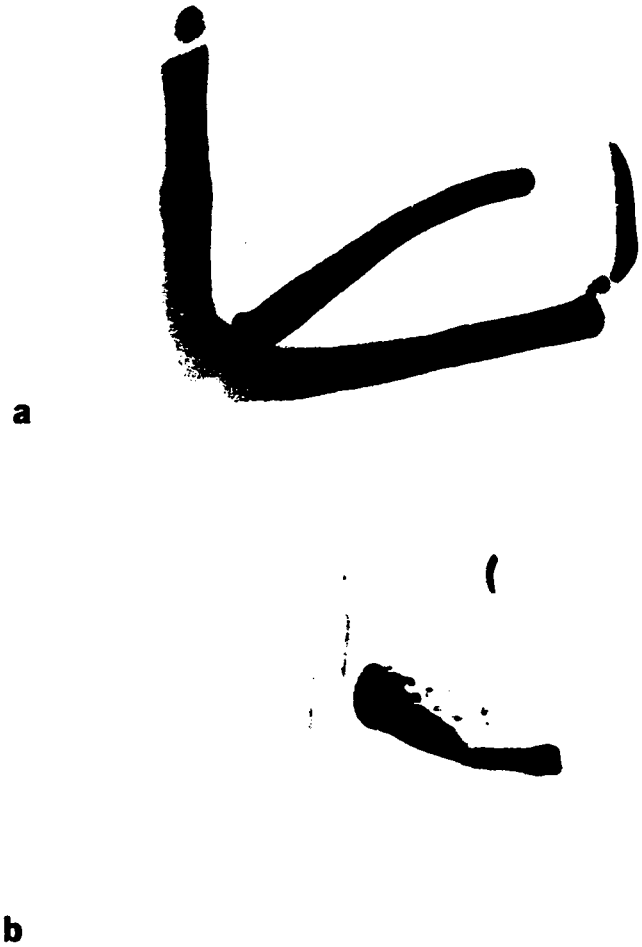


Figure 12. Proximal to distal transplant controls.

(a). Control host wing, amputated at junction of zeugopodium and autopodium. (b). Donor leg, amputated through the mid-femur.

Figure 13. Proximal to distal transplant graft limbs.

No regulation is seen in any graft; all have zeugopodia from both host wing and donor leg tissue. (a). Host and donor zeugopodia aligned at a right angle. (b). Donor zeugopodium inserted between radius and ulna of host zeugopodium. (c). Host and donor zeugopodia aligned side-by-side.

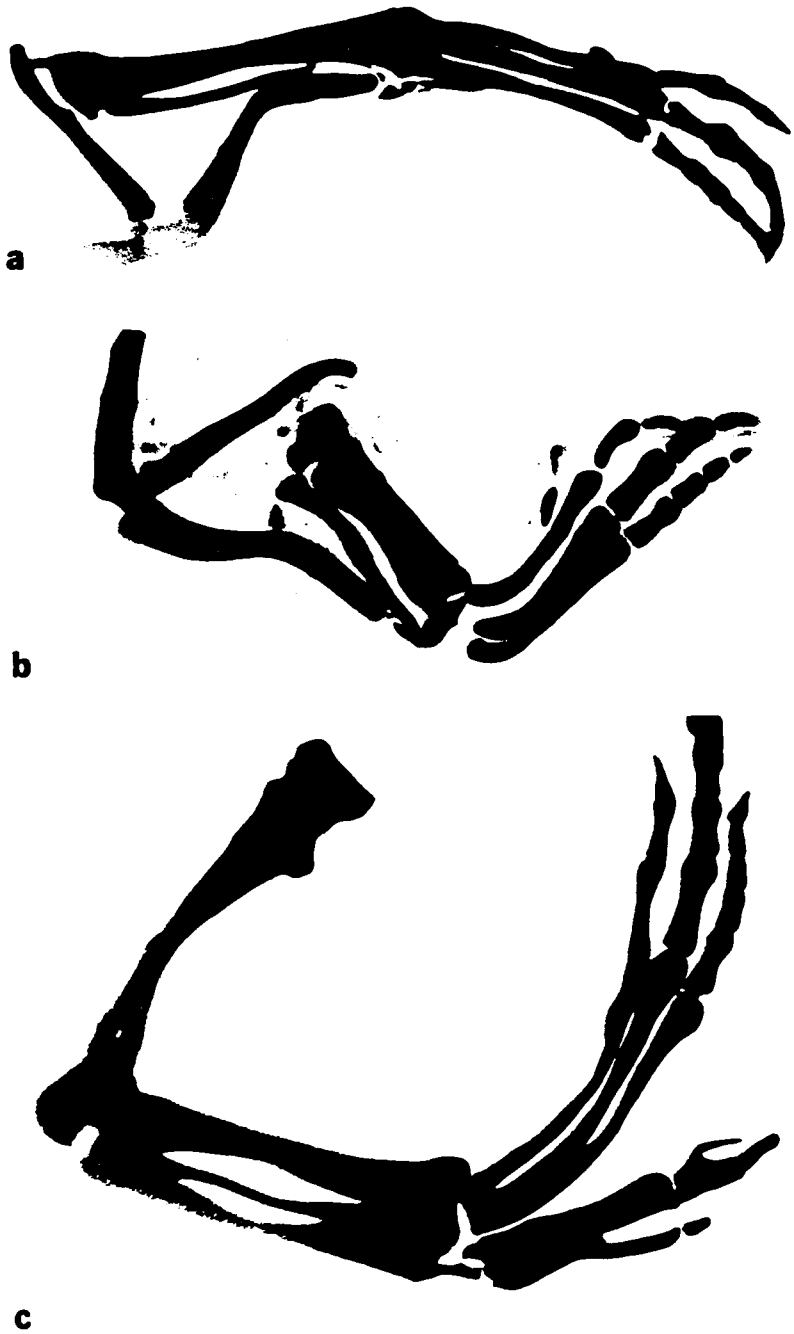


Figure 13.

Figure 14. Distal to proximal transplant controls.

(a). "Flank" group control host wing. No humerus is present. (b). "Humerus" group control host wing. About half the proximal humerus is present. (c). Donor leg. Amputated at the junction of the zeugopodium and autopodium.



a



b



c

Figure 14.

Figure 15. Distal to proximal transplant graft limbs.

(a). Donor foot and host shoulder; no humerus present. (b). Donor foot and proximal half of host humerus. (c). Donor foot and proximal half of host humerus. Note that distal end of humerus appears to be growing up towards the foot. (d). Donor foot and complete host humerus. (e, f and g). Donor foot and host humerus, where humerus is branching out to form "Y" structures. Increasing resemblence of the "Y" to a radius and ulna is apparent in this series. (h and i). Donor foot and host humerus, with a recognizable radius and ulna in between these. Regulation of presumptive deficiencies appears to be complete in these two grafts.



Figure 15.

APPENDIX II

TABLES

TABLE 1

D I F F E R E N T I A T I O N

Percent Cultures Showing First Myogenesis by Days
(Number Cultures Below Percent)

Explant:	APICAL					PROXIMAL				
Days: Stage	1	2	3	4	none	1	2	3	4	none
17	-	-	15 (3)	5 (1)	80 (16)	-	30 (6)	45 (9)	-	25 (5)
18	-	-	20 (4)	-	80 (16)	-	30 (6)	40 (8)	-	30 (6)
19	-	5 (1)	15 (3)	-	80 (16)	-	35 (7)	55 (11)	-	10 (2)
19 (- AER)	-	-	35 (7)	-	65 (13)	10 (2)	60 (12)	15 (3)	-	15 (3)
20	-	-	5 (1)	-	95 (19)	-	75 (15)	20 (4)	-	5 (1)
21	-	-	-	-	100 (20)	-	75 (15)	15 (3)	5 (1)	5 (1)
22	-	-	-	-	100 (20)	-	65 (13)	30 (6)	-	5 (1)
22 (- AER)	-	-	-	5 (1)	95 (19)	-	100 (20)	-	-	-
23	-	-	-	-	100 (20)	-	70 (17)	20 (4)	-	10 (2)

TABLE 2

PATTERN FORMATION

PROXIMAL TO DISTAL
(Presumptive Extra Segments)

Percent Limbs with Skeletal Structures
(Number Limbs After Percent)

Operation Resulting Structures	Stage	
	21	23
Host Wing Stump (Control)		
Fl	-	-
Fl, H	-	-
Fl, H, R-U	10 (2)	-
Fl, H, R-U, D	90 (20)	100 (29)
Donor Leg Stump		
part F	100 (11)	49 (19)
F	-	20 (8)
F, part T-F	-	26 (10)
F, T-F	-	3 (1)
F, T-F, A	-	3 (1)
Graft (Host/Donor)		
Correct Sequence		
Fl/F, T-F, A	-	-
Fl, H/T-F, A	-	-
Fl, H, R-U/A	10 (1)	-
Missing Segment		
Fl/A	-	-
Fl, part H/A	-	-
Fl, H/A	-	-
+ One segment		
Fl, H/F, T-F, A	-	-
Fl, H, R-U/T-F, A	10 (1)	42 (10)
Fl, H, R-U, D/A	-	-
+ Several Segments		
Fl, H, R-U/F, T-F, A	-	17 (4)
Fl, H, R-U, D/T-F, A	80 (9)	17 (4)
Fl, H, R-U, D/F, T-F, A	-	25 (6)

Symbols: Fl, Flank; H, Humerus; R-U, Radius and Ulua; D, Digits; F, Femur; T-F, Tibia and Fibula; A, Ankle and Toes.

TABLE 3

PATTERN FORMATION

DISTAL TO PROXIMAL
(Presumptive Missing Segment)

Percent Limbs With Skeletal Structures
(Number Limbs After Percent)

Operation / Resulting Structures	Stage				
	21 Fl	21 H	23 Fl	23 H	23/21 H
Host Wing Stump (Control)					
Fl	83 (15)	72 (13)	82 (18)	29 (6)	-
Fl, H	17 (3)	28 (6)	18 (4)	71 (15)	-
Fl, H, R-U	-	-	-	-	-
Fl, H, R-U, D	-	-	-	-	-
Donor Leg Stump					
Part F	-	-	-	-	-
F	-	-	-	-	-
F, part T-F	-	12 (2)	-	-	-
F, T-F	57 (13)	75 (12)	46 (17)	44 (8)	56 (9)
F, T-F, A	43 (10)	12 (2)	54 (20)	56 (10)	44 (7)
Graft (Host/Donor)					
Correct sequence					
Fl/F, T-F, A	-	-	-	-	-
Fl, H/T-F, A	6 (1)	8 (1)	-	-	-
Fl, H, R-U/A	25 (4)	46 (6)	-	31 (4) (all "Y")	87 (13) (4 "Y")
Missing segment					
Fl/A	25 (4)	15 (2)	28 (5)	-	-
Fl, part H/A	-	15 (2)	22 (4)	8 (1)	-
Fl, H/A	44 (7)	15 (2)	50 (9)	61 (8)	13 (2)

Symbols: Fl, flank; H, humerus; R-U, radius and ulna; D, digits; F, femur; T-F, tibia and fibula; A, ankle and toes.

VITA

Steven W. Gay was born and raised in Southern California. He received a degree of Bachelor of Science, with a major in Biological Sciences, at The University of California, Irvine, in 1976. He entered the Department of Zoology, The University of Tennessee, Knoxville, in 1977 and began graduate studies. While at The University of Tennessee, he worked as a Graduate Teaching Assistant and Graduate Research Assistant. He was awarded a degree of Doctor of Philosophy in Zoology in 1982 and has accepted a postdoctoral research position at The University of Connecticut Health Center in Farmington, Connecticut.