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Molecular and cellular correlates of myogenesis in the avian embryo

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Molecular and Cellular Correlates of Myogenesis in the Avian Embryo

by

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DISSERTATION

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DEDICATION

I wish to dedicate this dissertation to the inhabitants of 92-17 Vanderveer St., Queens Village, Thomas and Grace Williams, and to the inhabitants of 260-24 73rd Ave., Glen Oaks, Frederick and Dorothy Jeanes, citizens of a simpler time and place in New York City that no longer exists. The strength to carry this project to its completion was granted to me by my truly exceptional parents, Barry and Betty Williams. Their faith and sacrifices were always reliable and abundant; I can never repay them adequately. To Mimi Chiang, who has struggled along beside me during the past 10 years of my evolution as an academic scientist, I can only hope that the sacrifices that she has made have been worth the rewards. And to Georgianna, I hope that these first steps of the long journey into the future have put you in anticipation of great things to come. I know that they lead to magnificent places, and that one day you will be proud of what we have achieved as scientists at the dawn of the twenty-first century.

I would also like to express my gratitude to three people who have acted as mentor in my professional development. I would like to thank Karen Tanada Mills, who supervised my work as a beginning lab technician and who taught me to see accomplishment as something beyond the completion of an 8 hour work day. This was a very painful lesson to learn, but was administered humanely and graciously. I cannot thank her enough. Howard A. Bern, now Professor Emeritus at The University of California at Berkeley and a member of the National Academy of the Sciences, was a model of big-hearted generosity as the sponsor of my first independent research project and was instrumental in opening my eyes to the possibility of a career as a scientist. The appropriate word here is "mensch", and it is applied with profound respect and warm friendship. Finally, I wish to thank my advisor Charles P. Ordahl, who sponsored all of the work presented in this thesis and who knows how to get the best out of those lucky, unassuming, American kids from Heartland U.S.A. that end up in his lab. "In the shadow of the mighty oaks, the little acorns grow . . . .

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The coauthor listed in both of these publications directed and supervised the research which forms the basis for the dissertation.

ABSTRACT

Molecular and Cellular Correlates of Myogenesis in the Avian Embryo
Brian Andrew Williams

The molecular agents which cause different cell types to be formed in animal embryos are not understood. Factors that cause the formation of ectopic limbs and eyes have recently been described as "master regulatory genes", that direct large developmental programs of morphogenesis and differentiation. We have used experimental surgery techniques in combination with in situ hybridization and immunocytochemistry to test whether the myogenic basic helix-loop-helix (bHLH) transcription factors function as master regulatory genes to direct the decision of chick paraxial mesoderm cells to adopt the skeletal muscle fate. A method for marking the paraxial mesoderm prior to somite formation using the quail/chick grafting technique is described. The marked muscle precursor cells progress normally through subsequent events in specification such as the maturation of the somites, and migration into the nascent limb buds. In situ hybridization experiments with probes for Pax3, a paired-domain transcription factor related to the *Drosophila paired* gene, demonstrated that the Pax3 mRNA is expressed in muscle precursors of the somite dermomyotome and in migrating muscle precursor cells of the limb bud, prior to the expression of the myogenic bHLH factors.

An in vivo assay for cellular decision making was used to describe whether muscle precursor cells of the paraxial mesoderm and limb bud are determined to form skeletal muscle, prior to the expression of myogenic bHLH factors. Determined muscle precursor cells arise gradually in the dorso-medial somite and begin to appear at the onset of myogenic bHLH factor expression. Muscle precursor cells in the limb bud however, behave as determined skeletal muscle cells prior to the expression of myogenic bHLH factors, and during the time that Pax3 mRNA is expressed at high levels. We conclude that myogenic bHLH expression is a late event in the determination of limb skeletal muscle precursors, and that earlier events that are correlated with Pax3 expression in migrating mesenchymal cells derived from the paraxial mesoderm are sufficient for skeletal muscle cell determination.

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Introduction

Skeletal muscle development as a model for the study of cell type specification

I. General principles in the study of cell type specification

At the moment of fertilization, the zygote begins to fashion an organism comprised of many cellular descendants. The fusion of gamete nuclei into the diploid nucleus of the zygote is a unification of genomic material that will be maintained during many thousands of subsequent cell divisions. A characteristic of multicellular animals is a division of functions among different groups of cells, and integration of these functions to keep the organism alive in its environment. Therefore, the cells produced by the divisions of the fertilized egg at some point must begin to diverge from one another in appearance and function. This process of cell type specification leading to cellular differentiation has been a mystery to developmental biologists from the time human beings first looked into the causes of the generation of the animal form.

Today, developmental biologists recognize three distinct strategies employed by multicellular embryos for the establishment of cell types (Gilbert, 1994):

1) Syncytial specification is a strategy employed during early development of a well-studied animal embryo, *Drosophila melanogaster*. In this case, the zygote does not compartmentalize the egg cell cytoplasm during the first 13 divisions of nuclei. This creates a blastoderm consisting of a multinucleate syncytium. Prior to cellularization of the nuclei into blastomeres, the earliest events of specification occur. Nuclei in the future anterior and posterior ends of the embryo are exposed to a gradient of protein that is produced from messenger RNAs that are localized to the anterior and posterior ends of the egg cytoplasm (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988; Wang and Lehmann, 1991). After receiving these protein signals from the egg cytoplasm, the nuclei at each end of the egg enact transcription of specific genes that ultimately result in the assignment of axial identity along the rostro-caudal axis of the embryo.

2) Autonomous specification is used by embryos that undergo rigorously stereotyped patterns of early cleavage; well-studied examples are the nematode *c. elegans* and the embryos of ascidian larvae. A complete atlas of the nematode cleavage pattern from egg to adult worm is available; this pattern is known to be invariant. Nuclear and cellular divisions are coordinated in this type of development, so that each nucleus is situated in a compartment of the cytoplasm that is immediately separated from neighboring compartments by a cell membrane. Any communication between neighboring nuclei is mediated by the intervening cell membranes. During early cell divisions, molecular agents that are localized in different regions of the egg cell cytoplasm are segregated to each of the blastomeres. These agents begin the process of cell specification. For example, to properly specify cells that produce the nematode pharynx, the *skn-1* gene product is required during segregation of the early blastomeres (Bowerman et al., 1992). Antibody detection methods have demonstrated that it is segregated to separate blastomeres during early cleavage divisions in the embryonic worm (Bowerman et al., 1993). This strategy for development has been called "mosaic"; the name reflects the results of experiments in which some of the early blastomeres of the embryo are surgically isolated and develop according to their normal fate in the embryo (Whittaker, 1973).

3) Conditional specification is a strategy used by vertebrate organisms and may also be used by the urochordates and echinoderms (Driesch, 1893; Horstadius, 1939; Meedel et al., 1987). In this case, the specification process has been divided between signalling tissues and responding tissues. "Regulative" development, in which portions of the embryonic form are regenerated after surgical removal of some of the early blastomeres, is strong evidence that the communication of signals and some form of response to those signals, occurs in order to respecify cell fate. This idea is also encapsulated in the phenomenon of "induction", in which the appearance of differentiated cell types results from the experimental combination of signalling and responding tissues (Gurdon, 1987).

Inductive tissue interactions

Inductive interactions can occur as late events in the formation of organs (so-called proximate tissue interactions or secondary inductions). For example, the vertebrate eye is formed by a complex series of tissue interactions in amphibians and chicks (Grainger, 1992). Parenchymal organs of the urogenital tract require interactions between underlying mesenchymal cells and overlying epithelial cells to direct the differentiation of secretory and absorptive epithelium (Grobstein, 1955; Grobstein, 1956; Cunha, 1976b; Cunha, 1976a). Induction by proximate tissue interactions employs mesenchymal cells to direct the course of epithelial differentiation; there is also evidence that epithelial cells signal back to the mesenchyme. Positional information that dictates the outcome of differentiation may be encoded in the mesenchyme (Spemann and Schotte, 1932).

Inductive interactions are also seen much earlier in embryogenesis during the specification of the embryonic axis, the formation of the embryonic germ layers and the formation of various organ primordia. In the chick embryo, cells from the underlying hypoblast layer induce the direction of body axis formation in the overlying epiblast, prior to gastrulation (Waddington, 1932). In the early amphibian embryo, dorsal vegetal cells in the so called "Nieuwkoop center" induce "axis organizing" activity in the dorsal mesoderm (Nieuwkoop, 1969). A graft of dorsal mesoderm cells (the "organizer") to the future ventral side of the frog blastula is capable of recruiting ventral cells to dorsal mesoderm fates (Spemann and Mangold, 1924). The notochord which is formed by the cells of the organizer is required for the formation of the overlying neural plate in the late gastrula stage Xenopus embryo (Nieuwkoop and Nigtevecht, 1954), and later is responsible for the formation of specialized groups of cells in the floor plate of the neural tube (Placzek et al., 1990). Dorsal cell fates in the neural tube, particularly neural crest cell fate, appear to be induced by overlying ectoderm (Selleck and Bronner-Fraser, 1996). Patterning and the

specification of cartilage cell fates in the somite mesoderm are also induced by the notochord (Grobstein and Holtzer, 1955; Lash et al., 1957; Pourquie et al., 1993).

Molecular components of cell specification at the cell surface

Molecular influences on the specification of cell type can be conceptually divided between those at the surface of the cell membrane that interact with the embryonic microenvironment, and those which are confined to the nucleus, modifying gene transcription. Extracellular influences are sensed by specific receptors on the surface of the cell. The integrin family of receptors for example, are heterodimeric proteins inserted in the cell membrane which bind to different protein components of the extracellular matrix. They are linked through complex connecting proteins to the cytoskeletal actin network, and act as a target for the regulation of cell shape and motility. Integrin binding to extracellular matrix proteins can also regulate the ability of cells to differentiate (Menko and Boettiger, 1987). Other cell surface receptors function in the aggregation of "like" cell types during morphogenesis in early development. Cells that are specified to "like" fates express cell surface receptors (the cadherins) that form homodimers with identical receptors on the surface of "like"-specified neighbors (Takeichi, 1991). Matching cell surface profiles of cadherin expression during specification is a mechanism used for the coordination of individual cell fates into the formation of functioning tissue.

Signalling molecules or so called "growth factors" are soluble factors thought to diffuse through the extracellular matrix and can sometimes be bound to it. They are grouped in families by structural homology; examples include the transforming growth factor (TGF) family, the fibroblast growth factor (FGF) and epidermal growth factor (EGF) families. Receptors for signalling molecules are dimeric or monomeric proteins inserted in the cell membrane. Transduction of the signal from bound ligand through the receptor is often mediated through a kinase activity in the cytosolic domain of the receptor.

Targets for the kinase activity include the receptor itself, and other intracellular proteins that are components of complex intracellular signalling networks that use the regulation of phosphorylation for communication. The activity of intracellular signalling through these networks can lead to alterations in the regulation of transcription factor activity in the nucleus (Greenwald and Rubin, 1992).

Signalling molecules have been implicated in some of the very earliest events in embryogenesis leading to the specification of cell fate. A mechanism that appears to be used in promoting the specification of cell fates on the future dorsal and ventral sides of the embryo has recently been described in *Drosophila* and *Xenopus* embryos. TGF-B family members BMP-4 and *dpp* are thought to antagonize formation of central nervous system cell types, while homologs of the chordin/*short gastrulation* molecule are thought to promote the specification of central nervous system cell types (Holley et al., 1995). Importantly, the downstream events that lead to cell type specification appear to be evolutionarily conserved, since use of the *Drosophila* protein in *Xenopus* cells produces the same result as the *Xenopus* protein, and vice-versa. The mechanism by which soluble signalling molecules are capable of promoting the specification of multiple cell types has been the subject of intense investigation and much fruitful speculation. An imaginative proposal with many adherents is that soluble signalling molecules exist in a gradient radiating from a point of initial localization (Wolpert, 1971).

The gradient hypothesis: instructive or permissive signalling?

Cells that are subject to the specifying influence of signalling molecules distributed in a gradient are believed to be developmentally "naive" in that they have no predisposition to differentiate as any cell type in the absence of interaction with the molecule. In this case, the action of the signalling molecule is thought to be "instructive", and signalling to the responding cells is thought to be information rich. Different cell fates specified in response

to signalling are believed to result from exposure of the responding cells to different concentrations of the signalling molecule. In the gradient model, the spatial distribution of cell types in the embryo reflects the concentration of signalling molecule in any given position.

Whether soluble signalling molecules are actually distributed in a gradient and act in a way that is compatible with this notion is currently under close investigation. A soluble factor involved in cell type specification that may act in this way is activin, a TGF-B family member which has been best studied in *Xenopus*. Multiple cell types are formed when dissociated cells of the animal cap of the *Xenopus* blastula are bathed in different concentrations of activin (Green and Smith, 1990). Explanted *Xenopus* animal cap tissue responds to different concentrations of activin placed on beads by expressing marker genes at a distance from the bead corresponding to the concentration of activin used (Gurdon et al., 1994). Unfortunately, the gradient hypothesis is not supported by experiments in which epitope-tagged TGF-B and the TGF-B receptor are injected into separate cells of *Xenopus* animal cap explants (Reilly and Melton, 1996). In this case, the only receptor injected cells responding to injected TGF-B ligand are those in direct association with the ligand injected cells. Receptor injected cells at a distance from the ligand expressing cells do not respond by expressing marker genes characteristic of the distant response to TGF-B, and no epitope tagged TGF-B is detected on the surface of cells other than those initially injected. These results have been accepted as evidence in favor of a "relay" mechanism, in which cells respond to TGF-B receptor stimulation by secreting a secondary activator.

The case for cell type specification by signalling through a gradient of TGFB-like molecules has been made only indirectly in *Drosophila*. Marked clones of cells with activated forms of the *dpp* (a TGF-B family member) receptor are unable to produce a response of marker gene expression at a distance. Marker genes are only induced at a distance when functional *dpp* ligand is present. These results were taken as evidence that

some type of relay mechanism is not used for *dpp* signalling in the *Drosophila* wing disc (Nellen et al., 1996), but they are not a direct demonstration of a gradient of signalling molecule.

An alternative model exists to explain the phenomenon of induction by soluble factors. A "permissive" induction model attributes the appearance of different cell types to selective expansion of specified cells in response to the mitogenic effect of signalling molecules. In this model, responding precursor cells (or "founder" cells) for different cell types are believed to be specified at very early stages in development (Holtzer, 1978), much like the autonomous specification mechanism used by simpler animal systems. The earliest outward manifestation of their specification is a cell type specific mitotic response to the effects of signalling molecules. According to this model, the appearance of different cell types in different regions of the embryo reflects either a different spatial allocation of founder cells, or selective expansion of founder cells in response to localized production of lineage specific mitogens.

Molecular components of cellular specification within the nucleus

The process of cell type specification that leads to cellular differentiation is effected by the expression of subsets of the total genomic information available to each cell. In almost all differentiated cell types, the genomic information complement is as complete as that in the fertilized egg cell. This fact was demonstrated elegantly by transplantation of nuclei from differentiated cells of *Xenopus* intestinal epithelium into enucleated *Xenopus* oocytes. Although the experiment required several rounds of passage of nuclei through the early blastula stage, descendants of the transplanted nuclei were eventually able to produce adult frogs (Gurdon, 1962). Thus, while there appears to be no structural loss of the complete genomic information during development, the expression of this information is restricted to subsets of genes in differentiated cell types.

A molecular model for the expression of restricted subsets of genomic information in bacteria was initially proposed by Jacob and Monod (Jacob and Monod, 1961). A population of bacteria increase the expression of metabolic enzymes in response to inducing molecules in their environment. Genetic analysis revealed that in order for the "induction" response to occur, a locus separate from the structural genes coding the enzymes was required. Further analysis led to the conclusion that this locus coded for a separate molecule that acted in trans to repress the expression of mRNA from the structural locus coding for the enzyme. The action of the inducing agent was to prevent repression of the expression of the metabolic enzyme gene, by a direct or indirect effect on the activity of the repressor molecule. It is not entirely clear that the action of the repressor molecule was actually affected by interaction with the "inducer", in what might be described as a mechanism of "instructive" induction. Since the inducer molecule was an alternative source of essential nutrition, it is possible that it may have acted "permissively" to select for variants in the population of bacteria that had overcome repression of the metabolic enzyme gene by other means. These variants would expand in the population at the expense of organisms unable to compete for the alternative source of nutrients. The genetic evidence clearly demonstrated that a trans-acting repressor agent could be transferred from one organism to another during conjugation, and was encoded on a separate DNA locus. Thus, the existence of transcriptional regulatory molecules that controlled the expression of other functional proteins in the cell was demonstrated.

Further analysis of the molecular regulation of mRNA transcription has uncovered a variety of mechanisms that employ transcriptional activating proteins, cis-modifications of cytosine residues by methyl-directed methyltransferases (Sasaki et al., 1992), packaging of the DNA into nucleosomes and secondary structural features of the DNA itself. However, the regulation of the activation of gene transcription is only one control point in the restriction of the readout of the total genomic information available. Availability of mRNA

for protein production is regulated by other intracellular processes affecting message stability and initiation of translation. The proteins translated from mRNA molecules are also under regulation by degradative processes in the cell.

Specificity of transcriptional regulation within a lineage of cells and the acquisition of cell-type memory

The temporal progression of cells from the unspecified state to the fully differentiated state has been described as a lineage of inheritance relationships. Central to the idea of inheritance of cell type specification is the propagation of cellular identity through establishment of a mechanism for inherited cellular memory. At some point during progression through the lineage, a molecular mechanism is employed for the establishment of lineage identity. It is not clear whether the initialization of cellular identity results from the action of cytoplasmic and cell surface events, or from a nuclear event that is isolated from environmental influence. However, it is clear that a self-replicating structure inside the cell capable of providing temporal continuity will be involved in the maintenance of lineage memory in cellular descendants. The genetic material is the most likely candidate.

The discovery of lineage-specific transcriptional regulators in skeletal muscle cells has led to the proposal that direct or indirect auto-regulatory loops of transcription factor expression act to propagate cellular memory (Braun et al., 1989a; Thayer et al., 1989; Edmondson et al., 1992). In this model, transcriptional regulators perform a dual function by activating transcription of structural genes for proteins characteristic of differentiated cells in the lineage, and at the same time auto-activating transcription of their own (or related) mRNAs. Subsequently formed daughter cells receive these trans-acting factors during cell division and are able to maintain the program of gene expression.

Experimental analysis of stages in the specification of a cell lineage

The analysis of cells in a lineage prior to morphologically recognizable differentiation necessarily relies on cellular characteristics that are not immediately visible to the researcher. Typically, precursor cells in a lineage are described by their behavior in experimental assays. Several stages of precursor cell behaviors have been described at this point. Cells that are competent to differentiate do so under experimental conditions that are favorable for, or designed to elicit, the differentiation of the cell type in question. Cells that are specified for differentiation do so under experimentally "neutral" conditions, such as might exist in a simple tissue culture experiment. Cells that are determined to differentiate do so when grafted to regions of the early embryo where specification interactions for other cell types are occurring (Slack, 1983). By definition, determined cells contain some mechanism for the maintenance of cellular memory. Identifying the location and timing of appearance of cells in each of these specification stages is a necessary starting point for the identification of molecular mechanisms that are responsible for progression of cells through the stages of a lineage.

II. Skeletal muscle tissue as an experimental cell biology model

Striated skeletal muscles are one of the defining characteristics of organisms in the animal kingdom, organisms that are capable of self-initiated motion in physical space. The contractile ultrastructure of striated skeletal muscle tissue is conserved across the animal phyla. It consists of a repeating pattern of contractile units (sarcomeres) each of which is bordered by an array of Z-line proteins. Anchored to the Z-line proteins are the rods of myosin heavy chain molecules. The opposite ends of the myosin heavy chain molecules contain a catalytic domain for ATP hydrolysis, and a domain for calcium-mediated transient binding to neighboring actin filaments. The binding step is regulated by other sarcomeric

proteins, and ultimately results in the movement of the Z-line anchored myosin heavy chain molecules toward the center of each sarcomere. The end result is a contraction of muscle length, and the accomplishment of physical work.

The structure and transcriptional regulation of genes that code for the proteins of the sarcomeric apparatus is also highly conserved. These features have made the study of the ontogeny of skeletal muscle tissue an attractive model for understanding molecular regulation of the process of cell type specification during development.

Not all striated skeletal muscle tissue is equivalent. The molecular composition of muscle tissue imparts different physiological capabilities to each type of skeletal muscle. Slow and fast twitch muscle fibers, for example, are characterized by the presence of specific isoforms of myosin (Kelly and Rubinstein, 1980; Bandman, 1985). These isoforms are encoded by different genes, but the causes for their differential expression are not understood. In vitro analysis of colonies of muscle cells derived from early chick embryo limb buds has demonstrated that differential myosin isoform expression may be specified very early in development, and these differences may exist in individual muscle precursor cells (Miller and Stockdale, 1986).

In vitro methods for the study of muscle cell differentiation and the fusion question

To begin to understand the biology of the cells that form skeletal muscle tissue, a method for the cultivation of primary cells outside the body was necessary to isolate them from surrounding influences. One of the first in vitro cultivations of skeletal muscle tissue placed explants of developing muscle from chick embryos seven to eleven days old in Locke's solution where they were cultured for 3 or 4 days (Lewis and Lewis, 1917). The differentiated myofibers that grew out from the explants contained multiple nuclei within a syncytium. Cross-striations of the differentiated myofibers were described, and the multiple nuclei were observed carefully for mitotic figures; none were found. It was

postulated that the nuclei present in the the distal regions of the myofibers had migrated from the explants and may have undergone undetected mitoses while still in the explant. Also observed were mononucleate mesenchymal cells that were believed to give rise to the differentiated myofibers and so were called "myoblasts'. This was one of the first observations of muscle cells in a stage of specification that preceded overt differentiation.

Analysis of muscle cell differentiation was improved greatly when a method for the propagation in culture of single myoblasts and their expansion into colonies was devised by Irwin Konigsberg (Konigsberg, 1963). Addition of tritiated thymidine to myoblast cultures after differentiation demonstrated that the multiple nuclei resident within the multinucleate syncytium were no longer mitotically active (Bischoff and Holtzer, 1969), confirming Lewis' findings.

Whether muscle cell nuclei withdraw from mitosis immediately upon fusion into a syncytium, or whether they are slowly arrested over time was answered by an experiment in chimeric mice. Bea Mintz (Mintz and Baker, 1967) demonstrated that chimeric mice formed from two strains with subtle differences in the sequence coding for isocitrate dehydrogenase produced a chimeric form of this dimeric protein. The separate subunits were assumed to be translated and assembled in the same endoplasmic reticulum, a phenomenon that could only occur in syncytia containing nuclei from both of the parental strains. Syncytia that might form from the endoreduplication of nuclei during differentiation would be expected to produce dimers with identical subunits.

Myogenic cell lines

The development of cell lines whose differentiation could be synchronized and controlled by the withdrawal of serum factors from the medium allowed more detailed investigation into the nature of the cell biology of muscle differentiation (Yaffe, 1968). For instance, when cells propagated in calcium free medium were given the stimulus to

differentiate, it was found that fusion was prevented, although the expression of muscle specific proteins proceeded normally, as judged by detection with monoclonal antibodies (Paterson and Strohman, 1972). Thus, fusion is not required for the activation of transcription of muscle specific contractile protein genes. A small number of such differentiated myocytes subjected to the re-introduction of serum components into the medium can re-initiate mitoses, demonstrating that irreversible withdrawal from the cell cycle is not required for the expression of contractile proteins (Devlin and Konigsberg, 1983). More detailed experiments of this type, marking the cellular kinetics of mitotic withdrawal and the kinetics of onset of transcription of various contractile protein genes, provided a detailed analysis of the cellular physiology of muscle cell differentiation, and set the stage for the discovery of transcription factors that governed the process (Devlin and Emerson, 1978; Devlin and Emerson, 1979).

III. Experimental embryology and skeletal muscle ontogeny

Experiments in the embryo are required to understand the stages of skeletal muscle specification that are dependent on cellular interactions within the embryo. Although the intracellular organization of differentiated skeletal muscle cells is conserved across the animal phyla, experimental and genetic approaches reveal that there are a variety of strategies employed by different organisms for specification of the skeletal muscle lineage prior to differentiation. For instance, simple organisms such as *Drosophila*, nematodes, echinoderms and ascidians use a simplified genetic program for muscle specification. In all these organisms, only single copies of muscle specific transcription factor genes are found (Krause et al., 1990; Michelson et al., 1990; Venuti et al., 1991; Araki et al., 1994; Lilly et al., 1995). Cellular behaviors that generate differentiated skeletal muscles are also diverse. *Drosophila* produce a set of larval muscles for each of the body segments. Almost all of

these muscles are histolyzed during metamorphosis, however, a set of "founder" cells expressing the *twist* gene product are retained in each body segment that are believed to recruit other mesoderm cells to the muscle lineage by fusion (Bate, 1990; Bate et al., 1991; Rushton et al., 1995). Ascidian larva use both autonomous and conditional strategies for specification of the muscles of the larval tail (Meedel, et al., 1987). The ascidian oocyte contains a yellow cytoplasmic fraction (the myoplasm) that is segregated to blastomeres that are fated to form the larval muscle (Conklin, 1905). When transplanted to blastomeres fated to form the epidermis, the myoplasm converts these cells to the muscle fate (Tung et al., 1977; Whittaker, 1982), demonstrating that the segregation of cytoplasmic agents can direct the specification of skeletal muscle cells.

Conditional strategies for the ontogeny of skeletal muscle precursor cells in the vertebrates

Vertebrate embryos employ conditional strategies that require interaction among cells for specification of skeletal muscle precursor cells. Transcriptional regulation of the muscle specification pathway has evolved into a more complex network due to the duplication of ancestral genes for muscle specific transcription factors. The vertebrates have also developed more elaborate regulation of the activity of muscle specific transcription factors, employing mechanisms for subcellular localization, postranslational modification and alternative splicing of transcripts.

The first expression of muscle specific transcription factors seen in the zebrafish embryo occurs adjacent to the notochord in adaxial cells of the segmental plate (Weinberg et al., 1996). Adaxial cells are committed to differentiate as slow skeletal muscle fibertypes, and migrate from their position adjacent to the notochord to a more lateral, superficial region of the adult fish body (Devoto et al., 1996). Proper timing and spatial expression of muscle specific transcription factors is disturbed by mutations that affect the formation of the notochord (Weinberg, et al., 1996). Interference with signalling by *sonic hedgehog*, a

factor produced by the notochord, also causes gross aberrations in expression of muscle specific transcription factors (Hammerschmidt et al., 1996). Other hedgehog-related factors have recently been implicated in the formation of specific sets of muscle in the zebrafish (Currie and Ingham, 1996).

In the *Xenopus* embryo, unlocalized maternal transcripts for muscle specific transcription factors are contained within the egg (Harvey, 1990). Low level zygotic expression of these transcription factors is initiated in all cells of the embryo at the mid-blastula transition (Rupp and Weintraub, 1991), although expression of the protein is detectable only midway through gastrulation (Hopwood et al., 1992). The *Xenopus* embryo sequesters muscle specific transcription factors in the cytoplasm of cells that are not fated to form muscle (Rupp et al., 1994). After the induction of mesoderm in the animal hemisphere, up-regulated expression of muscle specific transcription factors becomes immunocytochemically detectable in the dorsal marginal zone of the mesoderm (Hopwood and Gurdon, 1990). Localization of transcription factors in cells of the dorsal marginal zone is nuclear (Rupp, et al., 1994). In this case, the establishment of muscle cell identity requires multiple interacting molecular mechanisms.

The relation between body segments and the differentiation of skeletal muscle cells is peculiar in *Xenopus*, when compared to other vertebrate embryos. The actual segmental units of the tadpole body plan are comprised entirely of unfused differentiated myocytes (Hamilton, 1969). *Xenopus* muscle precursor cells appear to have a requirement for interaction with neighboring cells that are undergoing the same process of specification and differentiation (Gurdon, 1988). Groups of skeletal muscle precursor cells from the early *Xenopus* gastrula can differentiate in the ventral body wall, but single cells from the same stage gastrulae cannot (Kato and Gurdon, 1993). Only in the late gastrula do skeletal muscle precursor cells acquire the ability to differentiate autonomously in this assay.

The chick embryo as an experimental embryological tool for the study of myogenesis

The embryonic chick is a useful experimental system to study the specification of skeletal muscle precursor cells in higher vertebrates. A well described series of developmental stages exists for gauging the window of time for experimentation, allowing meaningful comparison of results from different investigations (Hamburger and Hamilton, 1951; Hamburger and Hamilton, 1992). The chick embryo is accessible to surgical manipulation in ovo during the early stages of organogenesis, and can also be manipulated in culture during primary axis specification and gastrulation (New, 1955). Fragments of the embryo can easily be removed for culture in vitro or for ectopic implantation in ovo. Modern molecular biological methods are also being employed in this versatile system. The implantation of beads designed for the local release of soluble peptide factors has allowed experimentation with candidate factors involved in limb outgrowth and patterning (Riddle et al., 1993; Crossley et al., 1996). Replication defective retroviruses have been employed to produce localized overexpression of gene products involved in patterning the embryonic limb (Morgan et al., 1992). While targetted gene disruption is not yet available in this experimental system, the recent description of an embryonic stem cell line produced from the early blastoderm suggests that even this technology is not far off (Pain et al., 1996).

Formation and organization of the mesoderm in the chick embryo

Formation of the mesoderm, from which all skeletal muscle precursor cells arise in the chick embryo, has been carefully described by fate mapping experiments in New culture (Rosenquist, 1966; Schoenwolf, 1991). Mesoderm cells originate in the epiblast, a single layer of epithelium that contains all the cells that form the avian embryo. Cells from the underlying hypoblast imprint a direction for the future body axis on the cells of the epiblast; this orientation will later be realized as the direction of formation of the primitive streak (Waddington, 1932). Cells in the epiblast layer migrate towards this future midline and begin to coalesce as a thickening at the posterior midline of the future embryo. Ingression of the migrating epiblast cells occurs at this thickening, and they begin to form a layer of mesoderm and another of endoderm below the original epiblast (Vakaet, 1984; Bellairs, 1986; Eyal-Giladi et al., 1992). Continued migration of epiblast cells toward the midline causes the extension of this structure, the primitive streak, into a thin line of cells piled up in the epiblast at the future midline of the embryo. While the streak is being formed, cells continue to ingress forming the mesoderm and endoderm. The region of the epiblast that is fated to form the lateral plate mesoderm of the body wall migrates laterally. The region of the epiblast fated to form the paraxial mesoderm is deposited medially as it migrates through the streak. The process of mesoderm formation is completed by the regression of the rostral tip of the streak, Hensen's node, which sends the final component of the mesoderm, an axial structure called the notochord, through the streak.

The somites of the vertebrate embryo are the first morphological manifestation of the bilaterally symmetric, segmental nature of the vertebrate body plan. They are formed in a synchronous, pairwise fashion as epithelial spheres in the paraxial mesoderm. Cell matrix proteins characteristic of an intact epithelial tissue are produced by the paraxial mesoderm cells as they form into somites (Duband et al., 1987). Each pair of somites is

produced at intervals of approximately 100 minutes from the rostral tip of two blocks of mesenchymal tissue (the segmental plate) that lie adjacent to the notochord. The somites later undergo morphological transformations as they mature in a rostral to caudal sequence. The process of morphological maturation has been described in a staging system which assigns roman numeral I to the most recently formed somite from the segmental plate, II to the next rostral somite and so forth (Ordahl, 1993). The ventral epithelium of the somite becomes mesenchymal, and expands to surround the notochord and neural tube forming the sclerotome. Sclerotome cells are precursors for vertebral cartilage. The dorsal epithelium of the somite is retained and is referred to as the dermomyotome, to denote its dual fates as a source of precursor cells for the myotome and overlying dermis. The myotomal layer of the somite forms directly between the sclerotome and dermomyotome, and comprises the first differentiated skeletal muscle cells in the embryo.

A biological marking technique for cell lineage analysis in vivo

A powerful method for the analysis of cell lineage during development was introduced in 1969 by Nicole Le Douarin (Le Douarin, 1973). The surgical accessibility of the developing avian embryo is exploited for interspecific grafting of tissue fragments from the quail embryo into the chick embryo. After healing and further development, sections of the chimeric embryo are stained with Schiff's reagent, causing the nucleoli of quail cells derived from the graft to become visible under the microscope as brilliant crimson dots, compared to the pale staining nucleoli of the surrounding chick cells. All cells descended from the original graft are marked with the nucleolar marker, which is not transferable to the surrounding host cells. The grafted quail cells appear to faithfully recapitulate the development of the chick cells they are implanted to replace, although some concerns have been raised about differences in the rate of growth of grafted tissues (Noden, 1984). The development of derivatives of the neural crest (Le Douarin and Teillet, 1974) and the

ontogeny of the the hematopoietic system (Le Douarin et al., 1975) have been successfully described using this technique.

Discovery of the location of myogenic precursor cells in the paraxial mesoderm

A seminal advance in our understanding of the ontogeny of skeletal muscle in the chick embryo was produced by the efforts of Bodo Christ, using the quail/chick interspecific marking system (Christ et al., 1974); these findings were later confirmed in English (Chevallier et al., 1977; Christ et al., 1977). In an experiment designed to follow the development of the vertebral column, Bodo Christ replaced the somites at the future brachial level of the chick embryo with those of the quail. The vertebral cartilage and myotomal muscles that later form the intervertebral muscles were clearly populated by large numbers of quail cells. The startling result was that the entire limb musculature located distal to the body axis was also populated by quail cells. A population of somite derived cells had left their residence in the body axis and migrated out into the limb mesenchyme. At the time, limb muscle was widely believed to arise independently in the limb from mesenchymal cells that made developmental decisions between cartilage and muscle (Detwiler, 1955). Thus, a key step in the formation of the majority of skeletal muscle in the vertebrate body is the migration of somite cells away from the embryonic body axis. Continued experimentation with the somite grafting procedure showed that precursor cells for all of the body musculature are located exclusively within the paraxial mesoderm compartment, and are not found in other mesodermal compartments. The muscles of the face and eyes are produced by unsegmented paraxial mesoderm and prechordal mesoderm in the head (Johnston et al., 1979; Noden, 1983; Couly et al., 1992).

It is not yet known why skeletal muscle precursor cells are found only in the paraxial mesoderm and not in other more lateral regions of the mesoderm. Transplantation assays using portions of the primitive streak and segmental plate have been designed to

characterise the timing and location of this restriction of muscle specification. Cells that are competent to form skeletal muscle when grafted into the permissive environment of the limb bud reside in areas that correspond to the fate map for the paraxial mesoderm (Krenn et al., 1988). Other areas of the epiblast are not competent to form skeletal muscle in this assay. The establishment of competence appears to require some type of interaction or motion during gastrulation. If gastrulation is blocked by the application of a motility inhibitor (cytochalasin B), competence to differentiate as muscle is not observed (von Kirschhofer et al., 1994).

Morphogenesis of the myotome

Christ has also produced a description of the morphogenesis of myotomal muscle cells from specific regions of the somite dermomyotome (Kaehn et al., 1988). Using monoclonal antibodies to desmin, the location of the first differentiated muscle cells was described, and a model for their development from precursor cells in the dermomyotome was postulated. Cells at the cranial edge of the dermomyotome were thought to form the first myotomal cells, and as development proceeded, additional cells were recruited to the myotomal layer from progressively more lateral regions of the cranial edge of the dermomyotome.

This anatomical model for the generation of the myotome has recently been improved (Denetclaw et al., 1996), employing fluorescent cell labelling techniques to mark small groups of muscle precursor cells in the overlying dermomyotome layer. The first myotome cells to form are produced from both the medial and cranial edges of the dermomyotome. The cells elongate in cranial and caudal directions until they span the cranial/caudal length of the dermomyotome. New cells added to the myotome layer are deposited in the medial position, so that the oldest, first formed myotome cells eventually become displaced further laterally. This spatial arrangement suggests that a rapid growth of

the dermomyotomal precursor layer occurs medially, placing new precursor cells for the next myotome fibers more medially in relation to those already differentiated as myotome.

Limb muscle lineages in vitro

Precursor cells for the limb muscles vary in their requirements for soluble factors in order to survive and differentiate as skeletal muscle in vitro (White and Hauschka, 1971). They also change in respect to the size and shape of differentiated muscle cells derived from them. Limb buds collected from embryos at early stages of development were dissociated to monocellularity and plated at clonal density (White et al., 1975). While limb buds from all stages of development tested are able to produce colonies from single cells, the ability to differentiate as muscle is seen in some instances only with manipulations of the culture medium. Early limb bud stages tested (day 3-4) can produce colonies that differentiate as muscle only when cultured in medium that has been conditioned by the growth of other muscle colony forming cells. The muscle cells produced in these colonies are broad and short with few centrally located nuclei. Colonies from limb buds at intermediate and late stages of development form large, swirling colonies with hundreds of eccentrically placed nuclei per myotube. These later stages also differ in their ability to produce differentiated muscle cells after a brief exposure to fresh medium. Cells from late stage limb buds can differentiate in completely fresh medium. Finally, a subset of the fresh-medium-sufficient cells displays a requirement for the presence of nerve cells or nerve extract in the medium (Bonner and Hauschka, 1974; Bonner, 1978). It is not clear whether these different types of muscle colony forming cells are lineally related to one another, or whether they migrate into the limb as separate lineages from the somite.

Inductive interactions dissected in vitro

Simplified in vitro systems have been used to identify the minimal tissue interactions necessary to elicit muscle cell differentiation or the activation of muscle-specific gene transcription from explanted paraxial mesoderm (segmental plate or somites). The axial structures (notochord and neural tube) and the ectoderm overlying the somites have been implicated in the specification of somite cells to the skeletal muscle fate (Vivarelli and Cossu, 1986; Kenny-Mobbs and Thorogood, 1987; Buffinger and Stockdale, 1994; Fan and Tessier-Lavigne, 1994; Munsterberg and Lassar, 1995; Stern and Hauschka, 1995). The timing of the interaction between the axial structures and the paraxial mesoderm appears to be crucial. Segmental plate cells from early chick embryos (stage 10HH) are incapable of forming muscle unless co-cultured with both notochord and dorsal neural tube (Munsterberg and Lassar, 1995). As the somites are formed and mature, the requirement for notochord is lost; by somite stages IV-VI, co-culture with dorsal neural tube is sufficient to elicit myogenesis, although a weak inductive effect by notochord alone has been demonstrated (Buffinger and Stockdale, 1994). More mature somites from older embryos lose a requirement for tissue interaction altogether and produce skeletal muscle cells autonomously in culture (Buffinger and Stockdale, 1994; Munsterberg and Lassar, 1995; Stern and Hauschka, 1995).

The signals from the neural tube and notochord are soluble and can pass across a membrane to elicit muscle differentiation (Buffinger and Stockdale, 1995). Attempts to identify the specific molecules responsible for muscle specification have identified *sonic hedgehog* as a candidate for the notochord factor, and *Wnt* family members 1, 3 and 4 as candidates for the dorsal neural tube signal (Munsterberg et al., 1995; Stern et al., 1995). The timing and location of expression of each of these candidate molecules in the embryo is consistent with a role in signalling the specification of skeletal muscle from the paraxial mesoderm. The ability of other *sonic hedgehog* expressing tissues such as the floor plate

of the ventral neural tube to substitute for the notochord in the in vitro assay is further evidence for a role for *sonic hedgehog* as a molecular agent required by the somites (Munsterberg and Lassar, 1995). However, recent gene disruption experiments in mouse have shown that the absence of *sonic hedgehog* does not prevent the initial expression of muscle specific transcription factors, but does interfere greatly with the formation of muscle tissue (Chiang et al., 1996).

Skeletal muscle minimalism

In what may perhaps be the minimal system for assay of muscle differentiation in cells of the chick embryo, two groups have demonstrated that cells from the epiblast are capable of differentiation as skeletal muscle under appropriate conditions in vitro prior to gastrulation (Choi et al., 1989; George-Weinstein et al., 1996). These results conflict with the requirement for gastrulation described by others (von Kirschhofer, et al., 1994), and suggest that something in the culture conditions may mimic the inductive influences present during gastrulation. Differentiation of epiblast cells as skeletal muscle has been interpreted as evidence for a small population of muscle founder cells (Choi, et al., 1989), and also as evidence for a preferred pathway (or default pathway) for skeletal muscle differentiation in cells from the early embryo (George-Weinstein, et al., 1996). If the skeletal muscle differentiation program is in some sense "prior" or preferred to the enactment of other programs, it would suggest that this program may be actively repressed during early development until the morphogenetic events of embryogenesis have sorted precursor cells into the appropriate compartments for the formation of muscle tissue. The gradual appearance of skeletal muscle during development would then reflect a gradual disappearance of repressive mechanisms.

IV. Molecular regulation of muscle cell differentiation in vitro

Muscle specific transcription factors isolated the hard way

Our understanding of the molecular regulation of skeletal muscle differentiation has advanced rapidly due to the availability of methods for the in vitro cultivation of primary skeletal muscle cells and the availability of skeletal muscle cell lines where differentiation can be controlled by manipulation of medium components. Population analysis of mRNA during the differentiation of muscle cells in vitro has suggested that the onset of contractile protein mRNA transcription is coordinately regulated. Presumably, this would allow for the simultaneous production of proteins to be assembled in the sarcomeric apparatus (Devlin and Emerson, 1978; Devlin and Emerson, 1979). A simple transcriptional regulatory apparatus that is common to the many proteins required in the differentiated muscle cell could account for this coordinate regulation.

The regulation of contractile gene expression has been studied in vitro with modern molecular biological techniques. The identification of common cis-regulatory elements in the regulatory regions of contractile protein genes is routinely studied by transient transfection of reporter plasmids carrying muscle specific regulatory sequences (Mar and Ordahl, 1988; Sternberg et al., 1988). These elements are then used in electromobility shift assays and protein purification protocols as a method for characterizing and isolating DNA binding activities that may function in the regulation of transcription (Gossett et al., 1989; Mar and Ordahl, 1990). The cis elements can also be used as a probe for screening a *cDNA* expression library, and the DNA binding protein(s) can be cloned and purified for further analysis (Yu et al., 1992). With the cloned protein in hand, biochemical and mutational analysis of the protein can proceed (Molkentin et al., 1996a; Molkentin et al., 1996b). Interactions with other nuclear factors can then be tested, and the role of the

protein in activation or repression of transcription after transfection into skeletal muscle cells determined (Molkentin et al., 1995).

The discovery of myoD and myogenic conversion

While the use of primary skeletal muscle cells and myogenic cell lines in vitro have greatly aided the analysis of the differentiation process, earlier stages of specification in the skeletal muscle lineage have been more difficult to model in vitro. The first look into the earlier stages of specification was made possible by the discovery of a cell line (C3H 10T1/2) that can be chemically induced to advance from a non-specific mesodermal fibroblast phenotype to muscle, cartilage and fat precursor cells (Constantinides et al., 1977). Muscle precursor cells derived from the 10T1/2 cell line behave as a typical myogenic cell line in their requirement for serum withdrawal for differentiation. Thus a previously unidentifiable stage in the myogenic cell lineage, the "uncommitted mesodermal progenitor" was now available for in vitro experimentation.

Treatment of 10T1/2 cells with the DNA hypomethylating agent 5-azacytidine appeared to unlock a program of myogenic specification that was regulated by the cis mechanism of methylation of CpG islands (Konieczny and Emerson, 1984). DNA transfection experiments later showed that introduction of DNA from 5-azacytidine treated myogenic cells into untreated 10T1/2 cells could convert them to the myogenic lineage (Lassar et al., 1986; Pinney et al., 1988). Apparently, any trans-acting factors required for the conversion were already active in untreated 10T1/2 cells. Quantification of the chemically treated DNA for transfection suggested that the number of chemically modified genomic loci required to produce the myogenic conversion was small, possibly only one or a few (Lassar, et al., 1986).

Attempts to clone and characterize the genomic locus of the myogenic converting activity failed (Pinney, et al., 1988). However, subtractive cDNA libraries prepared from

treated and untreated 10T1/2 cells, produced a cDNA (myoD) which is capable of causing the myogenic conversion when constitutively expressed at high levels in a wide variety of primary cell types and cell lines (Davis et al., 1987; Weintraub et al., 1989; Choi et al., 1990). The muscle cells produced by this transfection appear to recapitulate the differentiation program completely; the timing of sarcomeric protein expression and the structure of the sarcomeres is indistinguishable from primary cells (Holtzer et al., 1991). Thus, MyoD was hypothesized to act as a "master regulatory gene" or "nodal point" in myogenic specification (Tapscott et al., 1988; Weintraub et al., 1991), responsible for directing the decision from uncommitted mesodermal progenitor cells to skeletal muscle cells.

Molecular characterization of the action of myoD, and the discovery of related molecules

The predicted amino acid sequence of the myoD cDNA identified a basic helix-loop-helix (bHLH) motif which is characteristic of the myc superfamily of transcription factors. The bHLH region is the only portion of myoD required for myogenic conversion (Tapscott, et al., 1988). In fact, a substitution of three amino acids in the basic domain of non-myogenic members of the bHLH family with three amino acids that are conserved among myogenic bHLH proteins is sufficient to impart myogenic converting ability (Davis and Weintraub, 1992). Deletion analysis identified the basic domain as the DNA binding and transcriptional activating domain (Lassar et al., 1989; Davis et al., 1990) and the HLH region as a motif which allows heterodimerization with members of the ubiquitously expressed E-protein family, a requirement for both transcriptional activity and myogenic conversion (Lassar et al., 1991). The consensus binding site for the myoD protein was identified as a six base core, CANNTG (or the muscle-specific E-box), (Lassar, et al., 1989) which is present in the upstream control regions of virtually every skeletal muscle specific gene yet described. Thus, conservation of the interaction between myoD and the

E-box for regulation of muscle gene expression is a common mechanism for the coordination of muscle gene expression during differentiation.

Since the isolation of myoD, three other myogenic basic HLH transcription factors have been identified. Myogenin, which appears to be involved in later steps of the muscle differentiation program, was isolated from the rat L6 myogenic cell line. L6 cells were treated with BrdU, a thymidine analog which prevents the differentiation of muscle cells by an as yet undescribed mechanism. Subclones of BrdU treated L6 cells were then selected based on their ability to overcome BrdU repression of myogenesis after withdrawal of serum from the medium. cDNA libraries were prepared from these cells and subtracted against a cDNA library from differentiation blocked L6 cells. A resulting clone was found to have a bHLH domain that is highly homologous to the myoD bHLH domain, and was shown to have myogenic converting ability in the 10T1/2 cell assay (Wright et al., 1989). The other members of the myogenic bHLH family, myf5 and MRF4, were isolated from muscle cell cDNA libraries using fragments of the myoD molecule (Braun et al., 1989b; Miner and Wold, 1990).

Repressors of myogenesis

Repressors of the myogenic function of the myoD family members have been identified. There appears to be negative regulation at several levels of activity of the protein. A conserved threonine residue at position 87 in the basic domain is a target for protein kinase C-mediated phosphorylation, which blocks the transcriptional activating ability of myoD (Li et al., 1992). This effect is apparently due to a loss of DNA binding activity by the phosphorylated form.

Id proteins were first identified as a set of truncated binding partners for the E-proteins. They contain a functional HLH domain which allows them to bind the E-proteins, but they lack the necessary basic domain required for DNA binding and

transcriptional activation. Negative control of myoD activity may thus be exercised by competition for required nuclear co-factors (Benezra et al., 1990).

Identification of proteins that bind directly to myoD was performed by interaction cloning using the yeast two hybrid system (Chen et al., 1996). In this case a novel factor, IMF (inhibitor of myoD function) was isolated. IMF acts by binding to the basic HLH domain of myoD, and preventing the transport of myogenic bHLH proteins to the nucleus. It may also interfere with the interaction with E-proteins.

The MSX-1 gene product, a mouse homolog of the *Drosophila* muscle segment homeobox (MSH) gene, has also been shown to inhibit myogenesis, but its effect appears to be exercised by negative regulation of transcription of myoD in the 10T1/2 cell assay. A putative MSX-1 binding site in the myoD core enhancer has been identified (Woloshin et al., 1995).

Homologs of two other Drosophila proteins, *notch* and *twist*, also have muscle repressing functions. *Notch*, when mutant in *Drosophila* embryos, results in the expansion of muscle cell colonies in mesoderm (Corbin et al., 1991). The mouse homolog of *Notch*, *Motch*, interferes with the myogenic activity of the myoD protein. Although *Notch* is a transmembrane protein, the cytoplasmic domain is cleaved and transported to the nucleus, where it interacts with the myoD bHLH region (Kopan et al., 1994). It is believed that this inhibition is a result of interference of myogenic heterodimer interactions with other unidentified nuclear cofactors that are required for transcriptional activation. Tethered dimers formed between myoD and E-12 are still inhibited by the action of *Notch*.

Myogenic repression by the *twist* protein, another non-myogenic bHLH factor, in mouse cells results from interference with at least three modes of myoD action (Spicer et al., 1996). DNA binding of the myogenic heterodimer is inhibited by *twist*. Interaction of the myogenic heterodimer with another muscle specific transcription factor, MEF2, significantly enhances the transcriptional activating ability of the myogenic heterodimer

(Molkentin, et al., 1995; Molkentin and Olson, 1996). This interaction is also blocked when *twist* is present. Finally, *twist* also interacts directly with the E-protein partners necessary for the myogenic activity of myoD, making them unavailable for heterodimerization.

Myogenic inhibiting function in cells of the uninduced *Xenopus* animal cap was demonstrated when co-injection of a *Xenopus* myf5 expression plasmid and a muscle specific reporter gene was performed. The coinjection resulted in the activation of the reporter plasmid, but no overt formation of differentiated skeletal muscle cells (Hopwood and Gurdon, 1990). The results of this experiment must be kept in mind, as it has become routine to describe trans-activation of reporter genes as evidence of "myogenesis".

V. Molecular hypotheses tested in vivo

The family of myogenic bHLH factors have been described as "master regulatory genes" (Weintraub, et al., 1991) which function in the specification of mesoderm cells to the skeletal muscle pathway. This handy appellation has been awarded to them based on their ability to heritably convert diverse types of primary and immortalized cells to the skeletal muscle fate, and on the behavior of 10T1/2 cells in response to chemical treatment with 5-azacytidine. That an immortal cell line can produce three distinct cell types in response to chemical treatment is remarkable, but does not necessarily confirm that this is a good model for the specification of cell types in the embryo. Nor should the results of forced expression of a transcription factor at high levels in cells be considered a true reflection of the role of that factor in normal cell function. The role of myogenic bHLH proteins in causing muscle cell differentiation by activating the expression of contractile protein genes is well established (Davis, et al., 1990; Edmondson and Olson, 1993). However, it is not at all clear how they might function to cause cellular decision making in

the mesoderm. One hypothesis invokes the establishment and maintenance of auto- and cross-regulatory loops of transcription factor activity. Transfection of cells with exogenous myoD results in the expression of endogenous myoD, as well as the expression of other endogenous myogenic factors (Braun, et al., 1989a; Thayer, et al., 1989; Edmondson, et al., 1992). Consensus E-box sequences in the regulatory control regions of members of the myogenic bHLH family have been identified (Goldhamer et al., 1992; Cheng et al., 1993; Yee and Rigby, 1993; Black et al., 1995; Goldhamer et al., 1995). Thus, while the maintenance of expression of myogenic family members may be explained by this type of mechanism, the initiation of the program only in specific cells of the embryo would seem to require an event independent of and prior to myogenic bHLH expression.

Myogenic factor expression in the embryo

The process of cell type specification is best studied in the embryo. If the myogenic factors truly do direct the decisions of mesodermal cells to acquire myogenic fate, their expression should be localized to regions of the embryo where these decisions are being made. The first application of molecular detection methods to the study of the muscle lineage in vivo was performed in 1957 (Holtzer et al., 1957). Light meromyosin (LMM) was detected in the myotome of the chick embryo using a polyclonal antibody. Now it is possible to detect the expression of specific messenger RNAs using radiolabelled or chemically substituted cRNA probes in the technique of in situ hybridization (Cox et al., 1984). Thus, molecules that have been shown in vitro to play a role in the specification of the muscle lineage can be localized in the developing embryo. Application of in situ hybridization technology to sections of the early chick embryo showed that expression of myoD mRNA was found in the somite myotome, and in the limb buds, co-incident with the expression of contractile protein mRNAs (de la Brousse and Emerson, 1990). Later studies which focussed on earlier stages of somite maturation found that the onset of

myogenic bHLH expression occurred in the epithelial somite of the chick at stage II of maturation (Pownall and Emerson, 1992). Other members of the myogenic bHLH family are expressed at different times during somite maturation, and in different populations of myogenic cells in the embryo, suggesting that they play different roles during embryogenesis (Sassoon et al., 1989; Ott et al., 1991; Pownall and Emerson, 1992).

Considerations of detection sensitivity are a concern when drawing conclusions about the role of locally expressed myogenic bHLHs in cell specification, and what type of mechanism may be responsible for the initiation of activation. RT-PCR methods that amplify myogenic bHLH transcripts show that low level expression is seen virtually throughout the embryo, and in cells that are not destined to form muscle (Rupp and Weintraub, 1991; Kopan, et al., 1994; Lin-Jones and Hauschka, 1996). This finding raises questions about the sufficiency of auto- and cross-activated myogenic factor expression for the establishment or the maintenance of the myogenic phenotype. It is possible that inhibitors are employed to keep low level expression of myogenic factors from effecting the outcome of cellular specification.

Experiments in transgenic mice

Transgenic mouse technology has developed to the point where some of the experiments that are possible in vitro can now be performed in the mouse embryo. Forced expression of myoD in the embryo causes the ectopic activation of skeletal muscle genes (Miner et al., 1992) and is ultimately fatal, yet formation of primary germ layers and the various organ primordia proceeds until mid-gestation (Faerman et al., 1993). Specification mechanisms for other cell types in the embryo seem to ignore the effects of the myogenic factors, especially at early stages of development.

Targetted gene disruption experiments have begun to shed light on the importance of the timing of expression of different myogenic bHLHs during development. A two

stage role for the myogenic factors is beginning to emerge, in which myoD and myf5 are believed to act earlier in the myogenic specification process than myogenin and MRF4 (Rawls et al., 1995). The inactivation of myogenin prevents the formation of differentiated muscle tissue, while presumptive myoblasts which can differentiate in vitro are present in the neonatal mouse (Hasty et al., 1993; Nabeshima et al., 1993). This two stage model has been supported by experiments with estrogen-inducible chimeric myoD molecules. Stably transfected cell lines cultured in cycloheximide respond to estrogen stimulation by expression of endogenous myogenin, but do not activate endogenous muscle creatine kinase or cardiac alpha-actin genes (Hollenberg et al., 1993).

Ablation of the early acting myogenic factors myoD and myf5 have uncovered separate roles for these two factors. Inactivation of the myoD gene causes up-regulation of myf5 expression, but has no observable muscle phenotype in the otherwise fully viable mice (Rudnicki et al., 1992). Inactivation of the myf5 gene has revealed a role in the morphogenesis of the myotomes, which appear to be required for the formation of ribs (Braun et al., 1992). The myotome is believed to have a previously unknown ability to signal the sclerotome through the localized secretion of FGF 4 and 6 (Grass et al., 1996). It has been argued that early expression of myoD and myf5 specifies two distinct lineages of myogenic cells in the embryo; myf5 expression begins in the medial half of the somite and may act to specify the myotome lineage, whereas myoD expression begins in the ventro-lateral region of the somite (Braun et al., 1994). These two populations of muscle precursor cells are produced by the differentiation of ES cells in culture. Selective ablation of the myf5 expressing lineage does not prevent the formation of the myoD expressing lineage (Braun and Arnold, 1996).

Combined inactivation of myf5 and myoD prevents the formation of differentiated muscle tissue and muscle precursor cells in the regions where they are expected to be found late in development. The mice die due to the lack of rib formation, but development

appears to proceed normally until birth. These results have been interpreted as evidence that expression of the early acting myogenic factors is required for the appearance of myogenic cells in the mesoderm (Rudnicki et al., 1993). Whether myogenic precursor cells in stages of the muscle lineage that precede expression of myf5 and myoD are present at early times during the development of these mice has not yet been explored. The results of these experiments should be interpreted in light of findings in simpler organisms. Inactivation of the single copy of the myogenic bHLH factor in *Drosophila* or in *c. elegans* does not prevent the formation of skeletal muscle (Michelson, et al., 1990; Paterson et al., 1991; Chen et al., 1992), demonstrating that molecular mechanisms independent of the myogenic bHLH factors can be employed to form skeletal muscle.

Regulation of myogenic factor expression

Phylogenetic comparisons of the structure of homeotic gene products have shown that structurally similar molecules can act to specify very different morphogenetic patterns. A conclusion has emerged that while the structure of transcription factors is crucial for their function in regulating expression of target genes, spatial and temporal regulation of transcription factor expression during embryogenesis provides much of the specificity of their action (Carroll, 1995). The implications of this conclusion for the regulatory control of myogenic factor expression have been recently examined. Gene swap (or "knock in") experiments in the mouse have inserted the myogenin coding region into the myf 5 locus. The mice are born and thrive with no apparent difficulties. The results clearly show that the rib forming function of myf5 expression is not unique to the structure of the myf5 molecule. Rather, the role of myf5 can be played by another myogenic bHLH, and is a result of the timing of expression of the gene to form myotome in the appropriate place at the right time (Wang et al., 1996).

Further understanding of the spatial and temporal control of expression of the myogenic bHLH factors will have to be approached by analysis of the trans-acting factors that regulate their expression. Transgenic mice carrying reporter constructs preserve the embryonic regulatory context and at the same time allow molecular dissection of transcriptional regulatory elements that drive expression of the myogenic genes. This technique has already been used to begin the characterization of minimal cis-acting elements required for correct embryonic expression of the four myogenic factors (Black, Cheng, Yee, Goldhamer, Goldhamer, Patapoutian).

VI. Summary of the research presented in this thesis

The experiments presented in this thesis were designed from the point of view that the quantum of meaningful research in the development of an organism is the cell. While there were many temptations to begin to study molecular interactions in a tightly controlled in vitro environment, the results of such experiments are significant only in so far as they are understood in the context of the cell. It will be a very long time before laboratory scientists understand the cellular environment and can perform truly meaningful molecular experiments. This is not to say that the detection of specific molecular species in the developing embryo is not important. Identification of the causative agents driving ontogeny requires this technology; it is here that our understanding of development will reach something approaching the certainty of the physical sciences. However, it must always be kept in mind that our certainty regarding the interaction of molecular species is always bought at the expense of performing experiments in a context that reflects the behavior of whole cells during the development of a complete organism.

The experiments presented here are an attempt to join the advantages of context with the accuracy of molecular biology by combining the methods of experimental embryology

with molecular detection techniques. I have deployed these procedures in an attempt to identify and characterize the timing and location of appearance of muscle precursor cells in the avian embryo and their behavior when they are in early stages of the myogenic lineage preceding differentiation and myogenic factor expression. This information will aid future researchers in their attempts to discover the mechanistic basis for myogenic memory in muscle precursor cells, and will help to interpret the results of myogenic gene disruption experiments in the mouse embryo.

Chapter 1 is a technical description of the methods for quail/chick surgery that were used in several of the later chapters. In this chapter, the entire paraxial mesoderm at the future brachial level of the chick embryo is marked by surgical replacement with the brachial paraxial mesoderm from a quail embryo at a similar stage of development. This procedure occurs prior to the time that several known steps in progression through the myogenic lineage have occurred, including the emigration of cells from the lateral half of the somite into the lateral plate mesoderm. The replacement quail cells are expected to undergo the early steps of somite morphogenesis and myogenic specification in synchrony with the schedule of the host cells on the unoperated side. A method for surgical ablation of the paraxial mesoderm is also presented, so that the effects of the paraxial mesoderm on surrounding tissues like the limb and body axis can also be judged. Surgical techniques used in other experiments can be found in the methods section of each chapter.

Chapter 2 is a description of a novel molecular marker that is expressed by muscle precursor cells from the time that they first migrate through the primitive streak. The Pax3 gene product is a transcription factor that was identified by homology to the *Drosophila* gene *paired*. It contains an octapeptide repeat sequence flanked by two DNA binding motifs, a paired domain which is characteristic of all 9 members of this gene family that have been identified in the vertebrates so far, and a complete paired type homeodomain. This arrangement of motifs is only found in Pax3 and Pax7 (Strachan and Read, 1994).

Pax3 expression is seen throughout the segmental plate mesoderm, and the pattern of expression is restricted to the precursor cells of the dermomyotome during the morphogenesis and maturation of the somites. Through the combination of experimental ablation of the paraxial mesoderm, and marking the paraxial mesoderm by replacement with a quail graft we were able to demonstrate that the gene is also expressed during the migration of somite cells into the limb mesenchyme. This expression precedes by two full days the expression of myogenic bHLH factors in these cells. Thus Pax3 is a bona fide marker for stages of myogenic specification that precede the expression of myogenic bHLH factors.

Chapter 3 describes a novel bioassay designed to identify the location and timing of irreversible decisions during the specification of muscle precursor cells in the dorso-medial paraxial mesoderm compartment. In this assay, dorso-medial paraxial mesoderm cells which are normally fated to form myotomal muscle and dermis are challenged to enter the cartilage pathway by exposure to excess notochord signals in a tightly constrained embryonic microenvironment. While most of the dorso-medial somite cells in this challenge assay are converted to the cartilage phenotype, a small population of determined muscle precursor cells begins to emerge as somites mature. This population becomes very large at later stages of somite maturation, and is capable of forming morphologically organized muscle tissue even when confronted with excess stimulus to form cartilage. The relationship between the timing and location of this behavior, and the timing and location of mRNAs thought to be involved in the muscle cell specification process are discussed.

Chapter 4 employs the assay from Chapter 3 to focus on decisions made during the specification of the precursors to limb muscles which arise from the dorso-lateral half of the somite (Ordahl and Le Douarin, 1992). Significant technical improvements are added to the experimental approach in this chapter. Chimeric limb buds containing quail muscle precursors are fed into the notochord challenge. The embryo has already "sorted" the

marked cells into a domain where they are fated to make muscle. In situ hybridization experiments in Chapter 2 demonstrated that the muscle precursor cells of the early limb bud express the Pax3 mRNA prior to overt expression of the myogenic bHLH factors. By dissociating the marked, Pax3 expressing muscle precursor cells of the limb from their neighboring mesenchyme, and then repacking the cells in a needle and feeding them back into the notochord challenge, we demonstrate that these cells are able to form only muscle and are not responsive to the cartilage inducing signals of the notochord. The cells are available as a suspension for part of this assay which renders them amenable to treatment with soluble factors, chemical agents and retroviral infection. We have tested whether the ability to form muscle is strictly due to the enhanced motility of migrating limb muscle precursors by immobilizing them with cytochalasin D. Even under these conditions, none of the cells join the cartilage tissue induced by the overlying notochords. This determined behavior of early limb muscle precursor cells is also seen if whole undissociated fragments of the chimeric limb are grafted into the notochord challenge. We have attempted to trace the origins of this determined behavior by placing dorso-lateral cells from the epithelial somite into the notochord challenge. In some cases, a small population of cells differentiates as muscle in the middle of the cartilage model. This behavior is never seen in grafts of the dorso-medial half of the somite, demonstrating that the muscle precursor cells in the medial and lateral halves of the somite differ not only in their spatial location, but also in their ability to form skeletal muscle when challenged with a stimulus to form cartilage. The sufficiency of myogenic bHLH factor expression for muscle cell determination is discussed and a new compartment in the specification pathway for skeletal muscle cell precursors is proposed.

CHAPTER ONE

Manipulation of the avian segmental plate in vivo

Introduction

The avian embryo provides a useful experimental system for the investigation of vertebrate development (Le Douarin and McLaren, 1984). Early events in avian development, such as axis specification, mesoderm induction and gastrulation, are usually studied in culture systems outside of the egg (Spratt, 1946; New, 1955; Vakaet, 1984; Eyal-Giladi, et al., 1992). However, once gastrulation is complete and the cells have begun to organize themselves into a recognizable, bilaterally symmetric vertebrate embryo, in ovo surgical experimentation can be carried out. In order to track the fate of experimentally manipulated cells in the embryo, cell labelling techniques are required. When combined with the tools of molecular biology, lineage tracing is a powerful tool for analysis of the mechanisms of cell specification and morphogenesis.

The quail-chick grafting method developed by Le Douarin (Le Douarin, 1973) has been successfully used as a cell labelling technique in the avian embryo. Organ anlage in the chick embryo are marked by surgical replacement with the corresponding anlage from a quail embryo. The cells generated by the grafted quail anlage can be identified in Feulgen-stained tissue sections by the presence of bright crimson nucleoli, which are absent from the pale-staining nuclei of host chick cells. This method allows long term analysis of the contribution of the graft, since the marker is transmitted faithfully to all descendant cells. An improvement in the resolution of the method has recently been introduced by the production of a monoclonal antibody (Carlson and Carlson, 1993) which allows the identification of individual graft-derived cells that are located in areas populated primarily by chick host cells.

The method presented here describes transplantation of fragments of the segmental plate (Ordahl and Le Douarin, 1992), the precursor to the somites, which are the embryonic anlage of skeletal muscle and the axial skeleton. Replacement of the chick segmental plate

with that of quail allows one to trace the development of skeletal muscle and the axial skeleton and to design experiments to analyze the specification of the cell types giving rise to these organs. Several different methods are presented, involving whole segmental plate replacement and extirpation as well as transplantation of selected fragments of the segmental plate.

Materials

Host and donor embryos

Quail eggs can be obtained in the USA from Strickland Quail Farm (Pooler, GA. Tel 912-748-5769). Chick eggs can be obtained from local producers. Eggs are stored at 4°C for up to 1 week and then incubated at 37-39°C in a humidified (60-80%) incubator.

Solutions

Tyrode's solution is available from Sigma Chemical Company (Catalog # T-2145) in powdered form, which should be stored at 4°C until reconstituted for use. Pancreatin is a crude enzyme preparation (Sigma Cat # P-3292) used to digest extracellular matrix in donor and host embryos. It is usually supplied at a 4X concentration, and should be stored in single use aliquots at -20°C as a 1X stock after dilution with Tyrode's. A 2% solution of fetal bovine serum (any supplier) diluted in Tyrode's is used to hold donor tissue fragments prior to implantation in hosts. Fetal bovine serum is stored in single use aliquots at -20°C until dilution with Tyrode's. Collagenase is stored in single use aliquots at 1% concentration in Tyrode's at -20°C, and is available from Sigma in powdered form (Catalog # C-0130).

Pipettes and pipette tips:

Mouth-operated micropipets are prepared by pulling borosilicate (not flint) glass microcapillary tubes (100 ul size; Fisher #21-164-28) over a small bunsen burner flame. The tips of pulled pipets are broken off to achieve the desired diameter, typically about 50-100 microns, or occasionally larger. Micropipets are connected to a mouthpiece using rubber or plastic hosing. For transporting donor tissue fragments, a P-20 pipetman or comparable tool is fitted with a narrow pipet tip (Phenix Research Products, phone #800-767-0665, cat #T-010BR).

Microscalpels

A method for creating tungsten microscalpels by electrolysis has recently been published (4). Tungsten wire can be ordered from Goodfellow (800-821-2870, Cat #005155; 0.38mm dia, 99.95% purity). We have found that during surgery, the tip of the microscalpel becomes coated with cellular debris from the incisions. It can be cleaned off by brief immersion in a small sonication bath. This method preserves the shape of the blade, while removing the adherent debris that dulls the microscalpel. Other microtools useful for embryo surgery, such as forceps, microscissors, insect pins and perforated spoons can be obtained from Fine Science Tools (800-521-2109).

Microdissection dishes.

Small glass concave embryological culture dishes (cat # 910B; Variety Glass, 614-432-3643) are filled halfway with black Dow Corning Sylgard (KR Anderson Co., cat # 170, black, 800-672-1858). The black Sylgard provides contrast for visualizing the white/translucent tissues of the embryo.

Sealing Tape

Windows and other holes in egg shells can be sealed with Scotch book mending tape available from Kielty and Dayton (catalogue # R8-191-CR).

Antibodies

The antibodies developed by Dave Bader (MF20) and Jean and Bruce Carlson (QCPN) were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242.

Methods

Preparation of donor embryos

Quail eggs are incubated round end facing up in a forced draft incubator at 37° C until stage 11-12 HH (approximately 48 hours). The egg is removed from the incubator and gently swabbed with a kimwipe soaked in 70% ethanol. Albumen is decanted through a small circular hole cut in the pointed end of the egg using a pair of curved scissors. After albumen decantation has lowered the embryo, the opening may be enlarged to facilitate further albumen decantation. The white stringy chalazae can also be cut with the scissors to facilitate decantation. The yolk, with the embryo on its surface, is then floated in Tyrode's solution contained in a small bowl (Figure 1.1A). The blastodisc is cut away from the yolk using a serrated forceps and iridectomy scissors. The embryo is removed to a small dissection dish with a perforated spoon, rinsed free of yolk platelets, and pinned to the Sylgard with 0.15 mm insect pins, ventral side up. The first pin is inserted through the *area opaca*, and then the opposite corner of the *area opaca* is pinned, after lightly stretching the embryo. Additional pins are then similarly inserted until the embryo is pinned in at least four corners (Figure 1.1B).

Removal of donor segmental plate.

Figures 1.1C and D illustrate the sequence of steps used to prepare the donor segmental plate. The notochord and underside of the early somites and segmental plate should be visible. A midline incision is made in the endoderm (Fig. 1.1C) parallel to the notochord using a short snipping stroke, where the scalpel tip is inserted into the endoderm and sharply lifted upwards (Ordahl and Christ, 1996). A small amount (< 5ml) of 1x pancreatin is pipetted onto the incision using a micropipette. Only a minimal amount of enzyme should be used, to prevent overdigestion of the donor tissue (see Critical Aspects, below). The digestive action of the pancreatin allows the endoderm, and any other tissues such as the aorta, to be teased away from the underlying segmental plate mesoderm. Once fully exposed, an orientation mark should be placed on the segmental plate using fine animal carbon or a vital dye or other method (Ordahl and Christ, 1996).

The first incision in the mesoderm is begun at the rostral tip of the segmental plate between its lateral margin and the medial margin of the Wolffian duct (Fig. 1.1D, step 1). The incision is extended caudally for a length equivalent to approximately 5 somites, using a slashing motion with either a microneedle or a broad, flat bladed microscalpel (Ordahl and Christ, 1996). This incision separates the segmental plate from the intermediate and lateral plate mesoderm, and gives a smooth lateral edge to the donor tissue. Next, the segmental plate is separated from the neural tube, first by scoring between the medial margin of the segmental plate and the neural tube, and then by teasing the segmental plate laterally away from the neural tube (Fig. 1.1D, step 2). Finally, a caudal transverse incision is made in the segmental plate at a point approximately 5 somites distance caudal to the rostral tip of the segmental plate (Fig. 1.1D, step 3). The segmental plate donor fragment can then be lifted away from the underlying ectoderm with the flat edge of the scalpel blade. If

necessary, additional pancreatin can be introduced to speed release of the segmental plate from the ectoderm.

The donor segmental plate is transferred to a holding dish using a P-20 pipetman. The pipetman allows the tissue fragment to be contained in a small, manageable volume of transfer solution. The pipet tip is first fully charged with 2% fetal calf serum to prevent tissue fragments from sticking to the internal surface of the tip. The tissue should not be drawn deeply into the pipet tip so that it can be easily moved in and out of the pipet by action of the plunger. The tissue fragment is then ejected into a droplet of 2% fetal calf serum in a small plastic culture dish, covered and stored at room temperature until transplantation into the host embryo (Figure 1.2E).

If the medial and lateral halves of the segmental plate are to be transplanted separately, the first incision should be made directly through the longitudinal midline of the segmental plate (Figures 1.3A and 1.3B), using the same slashing stroke described above, thereby dividing the medial and lateral halves of the segmental plate. After orientation marking, the lateral and medial halves are excised, respectively, using the scalpel strokes described in Figures 1.3A and 1.3B. The donor half-segmental plate fragments are then separated from the ectoderm and placed in holding dishes as described above.

Preparation of host embryo

Chick host eggs are incubated on their sides for 42-54 hours, to stage 11-12 HH (Hamburger and Hamilton, 1951; Hamburger and Hamilton, 1992), and are carefully maintained in this orientation thereafter. The top of the egg shell is marked to indicate the location of the embryo. The egg is removed from the incubator, swabbed with 70% ethanol and a small puncture is made in the pointed end by tapping with blunt forceps. An 18 gauge needle fitted to a 10 ml syringe is used to withdraw 0.5-2 mls of albumen through this hole, taking care not to puncture the yolk (Figure 1.2A). The removal of albumen

lowers the embryo away from the egg shell, allowing a window to be cut in the shell over the embryo with curved scissors. At this stage of development, the embryonic blood cells have begun to form and should be barely visible as a small red crescent at the posterior margin of the embryo. Ideally, the embryo will be centrally located on the surface of the yolk; if it is located grossly eccentrically, it is not a good candidate for surgery and should be discarded, and another host prepared. These steps are summarized in Figure 1.2A.

Under the dissecting microscope, the translucent embryo is almost invisible against the yellow background of yolk. A contrast medium prepared by mixing Pelikan #17 black ink 1:1 with Tyrode's solution is therefore injected between the embryo and the underlying yolk, using a fine tipped mouth pipette. The pipette, preloaded with contrast medium, is inserted through the vitelline membrane and the *area opaca* . After positioning the pipette tip under the embryo, a minimal amount of ink is expressed by mouth pressure. Once visible, the embryo can be staged precisely by counting the number of somites (Ordahl and Christ, 1996).

An small incision or puncture is made in the vitelline membrane near the head region of the embryo. It is then moistened with a few drops of Tyrode's solution applied with a Pasteur pipette. As the Tyrode's solution flows down through the incision, the vitelline membrane will float away from the embryo proper, and can be removed with forceps or a few strokes of the broad microscalpel, without damaging the embryo below.

Removal of host segmental plate

The sequence of incisions made in the host embryo is outlined in Figure 1.2 (B through E). The first incision is made by snipping the ectoderm between the segmental plate and the neural tube. This incision should extend farther cranially and caudally than the intended target site in the mesoderm (Fig. 1.2B). A small amount of pancreatin is

pipetted onto the longitudinal incision, and the flap of ectoderm teased away from the underlying mesoderm.

Incisions in the mesoderm are then made to circumscribe the portion of the segmental plate to be removed (Figure 1.2C, step 1). The region to be excised is then macerated using a microscalpel, taking care not to puncture the endoderm and blood vessels residing immediately below (Figure 1.2C, step 2). A minimal amount of pancreatin (<5 ml) can be introduced to loosen the fragments. The macerated tissue fragments of the segmental plate are removed by aspiration (Figure 1.2D), and the area is quenched with about 20 µl 10% fetal calf serum and rinsed three times with 20 µl of Tyrode's. When removal of the host segmental plate fragment is complete, the smooth surface of the endoderm should be visible at the bottom of the excavated area. If any bits of host segmental plate

adhere to the surface of the endoderm, they should be removed by aspiration.

If only medial or lateral halves of the segmental plate are to be replaced with quail donor fragments, the sequence of incisions is copied from Figures 1.3A and B. The figures show the approach from the ventral side of the donor, whereas removal of host tissue is performed from a dorsal approach. The temporal sequence of incisions is the same.

Implantation of donor segmental plate

The quail donor segmental plate fragment is transferred from the holding dish onto the surface of the host embryo blastodisc using a P-20 pipetman. The pipetman tip should be pre-charged with Tyrode's solution to avoid co-transfer of excess serum which can cause tissue fragments to "float" in the host environment. As necessary, the donor tissue fragment can be sized and trimmed to fit the excavated area of the host using a thin microscalpel. Taking care to preserve the orientation of the tissue as marked previously,

the donor graft is then gently tucked into position using the flat edge of a microscalpel (Fig. 1.2E). The ectoderm flap is replaced, and the egg is sealed with a pliable brand of tape (see materials). Taping the window closed on the spherical surface of the egg shell will cause pleats to form in the tape that should be sealed together tightly and pressed tightly to the egg shell to prevent loss of moisture from the embryo in the incubator. After a tight seal has been produced, the egg is returned to the incubator.

If ablation of the segmental plate is to be performed (summarized in Figure 1.3C and D), a "stuffer-fragment" should be inserted into its place to prevent the remaining posterior portions of the segmental plate from expanding into, and compensating for, the extirpated region. We have used stuffer fragments derived from both the lateral plate mesoderm and the acellular egg shell membrane. These provide a partial block to the invasion of cells from other regions of the mesoderm.

Lateral plate mesoderm is the more difficult of the two to prepare for grafting because it has an "elastic" quality that makes it difficult to slice with a microscalpel. It is most easily cut by slashing with a microneedle or a broad, flat microscalpel. The lateral plate fragment is then bathed in a few microliters of 0.5% collagenase to separate the ectoderm from the mesoderm. Once the ectoderm has been removed, the enzyme action is quenched with fetal calf serum, as above, and the mesoderm trimmed to fit.

Egg shell membrane can be prepared from the shards around the window in the host embryo shell. This tissue is impossible to cut with a scalpel, and should be trimmed using iridectomy scissors. It should be kept soaked in Tyrode's at all times to aid in positioning it into the excavated host site. After implantation of either type of stuffer fragment, the ectoderm is replaced over the operated site, and the egg is sealed as above and returned to the incubator.

Harvesting experimental embryos.

The re-incubation period (a few hours to several days) for chimeric embryos depends upon the research objective. The embryo is harvested by first carefully opening the window and irrigating the embryo with fresh Tyrode's solution. The embryo can then be cut away from the underlying yolk using a pair of serrated forceps and iridectomy scissors. It is then lifted out of the egg shell with a perforated spoon, and removed to Tyrode's solution in a dissection dish and rinsed free of yolk platelets. The Tyrodes solution is then withdrawn and replaced with fixative. The embryo is then processed for histological examination using standard procedures.

Critical aspects of the procedure

Host selection is an important factor for the survival of segmental plate grafts for long incubation periods. Embryos that are centered on the yolk, have evidence of blood island development at the posterior margin of the blastodisc, and a vigorously beating heart are the best candidates. The host should be out of the incubator for a minimum amount of time during surgery, ideally no longer than 20 minutes.

A thorough rinsing of the areas treated with enzymes is essential for good survival. We first quench such areas with 20 µl of fetal calf serum and then rinse at least three times with 20 µl Tyrode's solution. Rinsing is performed by flooding the surgery area with Tyrode's solution, followed by immediate removal by aspiration. If large holes inexplicably appear in the endoderm, or if the host has the appearance of being split apart in the region of the incision and excavation, it is possible that too much (or too long a duration of) enzyme has been used.

Finally, it is crucial that the window in the egg shell be resealed tightly with tape prior to re-incubation. Dehydration in the forced draft incubator is the major cause of death

in post-operative embryos, and this usually results from the tape not being adequately secured to the egg shell. If necessary, a second layer of tape can be placed over regions that are poorly sealed.

Results and Discussion

Figure 1.4 shows the distribution of quail cells in a chimeric embryo 3 days after the thoracic segmental plate of a chick host was replaced by that of quail. The contralateral side was unoperated and serves as a control. Panel A shows a section with dark blue quail nuclei stained with the anti-quail antibody, QCPN (Carlson and Carlson, 1993). Since the section is viewed from its cranial aspect the quail cells appear on the left side of the photograph in A. The entire paraxial mesoderm compartment is populated with quail nuclei on the operated side but none appear on the unoperated side indicating cells do not cross the mid-line of the embryo (arrowheads).

Panel B shows an adjacent section stained with the anti-muscle myosin antibody, MF20, to show developing muscles. The myosin positive cells (arrow) on the operated side of the section contain quail nuclei as shown in panel A. This type of analysis allows for the evaluation of the fate of cells derived from the grafted segmental plate.

Conclusions and Perspectives

The paraxial mesoderm of the avian embryo undergoes several morphological transitions during development, each of which has significance for the production of specified cell types, and for the organization of the body plan. A method for manipulation of the paraxial mesoderm after it has been divided into somites has recently been published (Ordahl and Christ, 1996). The methods described here allow analysis of the paraxial mesoderm prior to segmentation and invasion by neural crest cells from the neural tube,

and prior to events thought to be involved in the specification of cell lineages, as determined both by previous experimentation (Aoyama and Asamoto, 1988; Christ et al., 1992; Ordahl and Le Douarin, 1992) and by in situ hybridization studies (Pownall and Emerson, 1992; Williams and Ordahl, 1994). The paraxial mesoderm imposes pattern on the central nervous system, and therefore contains significant information for producing the segmented nature of the vertebrate body plan. In addition, the elements of the axial skeleton, as well as the entire musculature of the body, are produced from this tissue. Cell marking experiments allow the researcher to assess the contribution of marked cells to the future organs formed from the segmental plate, whereas the extirpation technique allows an evaluation of the effect of the segmental plate on surrounding tissues.

Figure 1.1. Preparation of donor embryo

(A) Removal of the donor embryo from the egg.

(B) Donor embryo staked out in Sylgard dish.

(C) Ventral view of the paraxial mesoderm of the donor embryo pinned in a Sylgard dish. An incision is made in the endoderm along the midline (pictured as a dotted line), and the endoderm is reflected away from the paraxial mesoderm after application of pancreatin.

(D) Ventral view diagramming the cuts in the donor mesoderm (pictured as dotted lines). The first cut (1) is made between the segmental plate and the intermediate mesoderm, for a length of about 5 somites. The second cut (2) is made between the segmental plate and the neural tube, and mainly involves teasing the segmental plate away from the neural tube. The third cut (3) is made transversely, approximately 5 somites distance caudal to the anterior tip of the segmental plate.

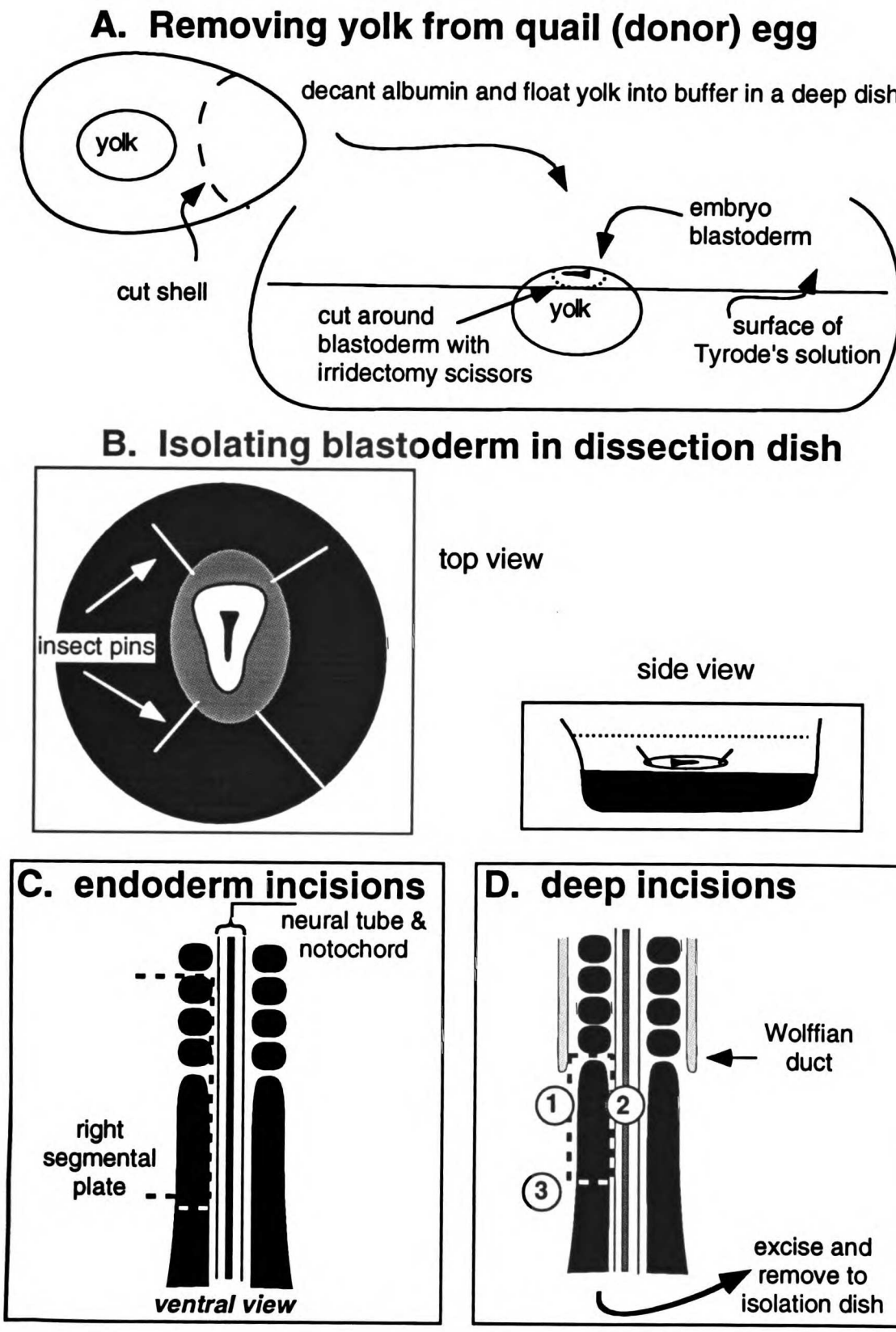
A. Removing yolk from quail (donor) egg
decant albumin and float yolk into buffer in a deep dish
yolk
cut shell
embryo
blastoderm
yolk
cut around
blastoderm with
irridectomy scissors
surface of
Tyrode's solution
B. Isolating blastoderm in dissection dish
insect pins
top view
side view
C. endoderm incisions
neural tube &
notochord
right
segmental
plate
ventral view
D. deep incisions
Wolffian
duct
1
2
3
excise and
remove to
isolation dish

Figure 1.1

Figure 1.2. Preparation of host embryo

(A) Scheme for lowering the host embryo from the shell.

(B) The first incision in the host ectoderm is made between the neural tube and the segmental plate, as indicated by the dotted line.

(C) Incisions in the host mesoderm. A longitudinal incision (1) is made lateral to the segmental plate: the tissue is then macerated (2) using a microscalpel.

(D) The macerated fragments are aspirated with a small mouth pipette.

(E) The donor tissue is moved into place with a microscalpel.

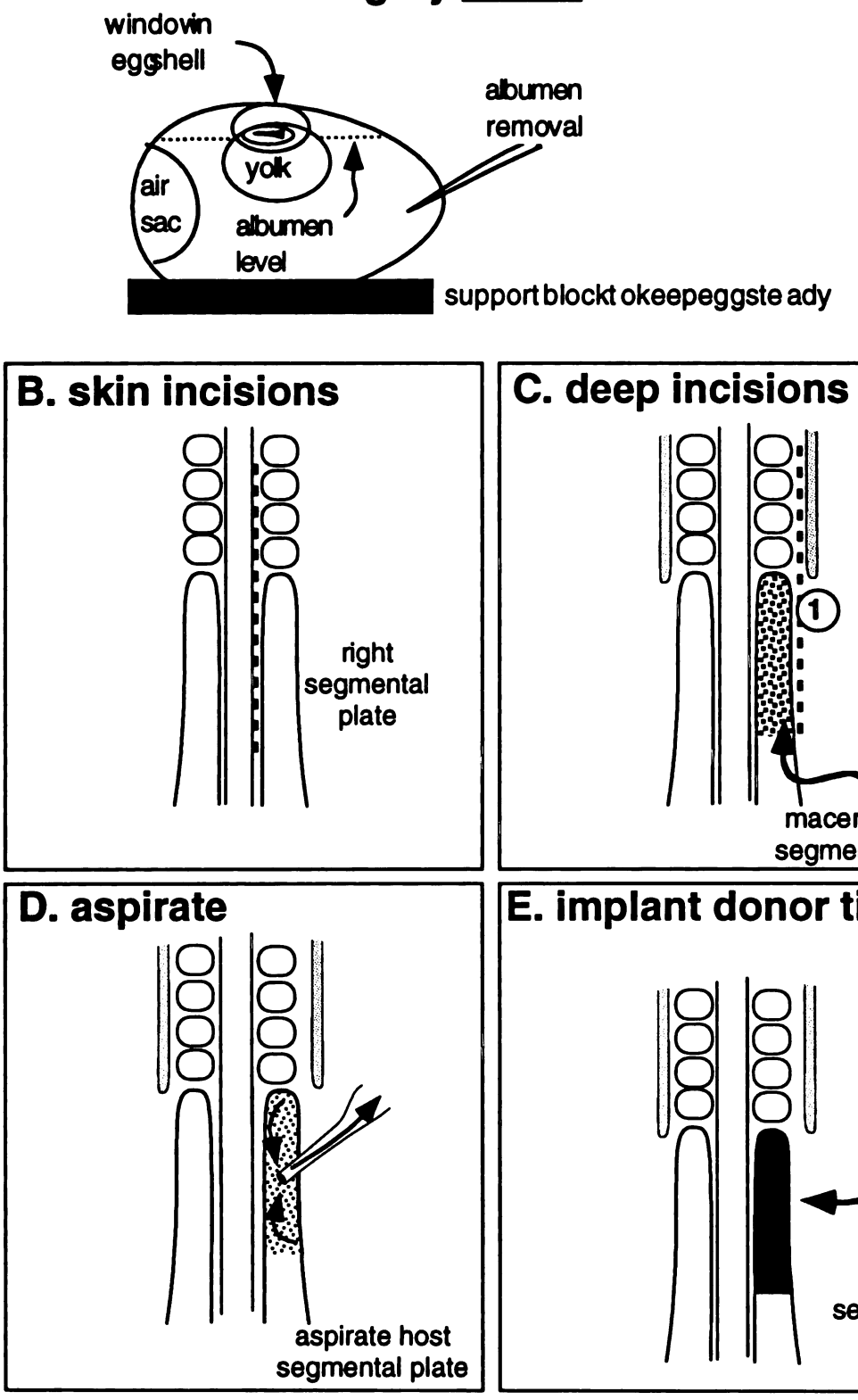
A. Positioning chick (host) embryo for surgery in ovo.
windowin eggshell
albumen removal
yolk
air sac
albumen level
support blockt okeepeggste ady
B. skin incisions
right segmental plate
C. deep incisions
1
2
macerate host segmental plate
D. aspirate
aspirate host segmental plate
E. implant donor tissue
quail segmental plate

Figure 1.2

Figure 1.3. Half segmental plate grafts and extirpation experiments.

(A) Ventral view diagramming cuts in the donor mesoderm for a lateral half graft. After reflection of the endoderm as in Figure 1C, a longitudinal incision is made in the center of the segmental plate for 5 somites distance. Next, a longitudinal incision is made between the intermediate mesoderm and the segmental plate, (2), followed by transverse incisions (3) and (4) which define the cranial and caudal extent of the graft.

(B) To prepare a medial half graft, the first incision (1) is again made in the middle of the segmental plate, followed by (2) an incision between the plate and neural tube, and finally transverse (3 and 4) incisions.

(C) Dorsal view diagramming preparation of the host for segmental plate extirpation. After incision of the ectoderm as in Figure 2B, and maceration of the segmental plate as in Figure 2C, the entire segmental plate is aspirated with a mouth pipette.

(D) A stuffer fragment prepared from either the host egg shell membrane or lateral plate tissue from a quail donor is used to replace the extirpated segmental plate.

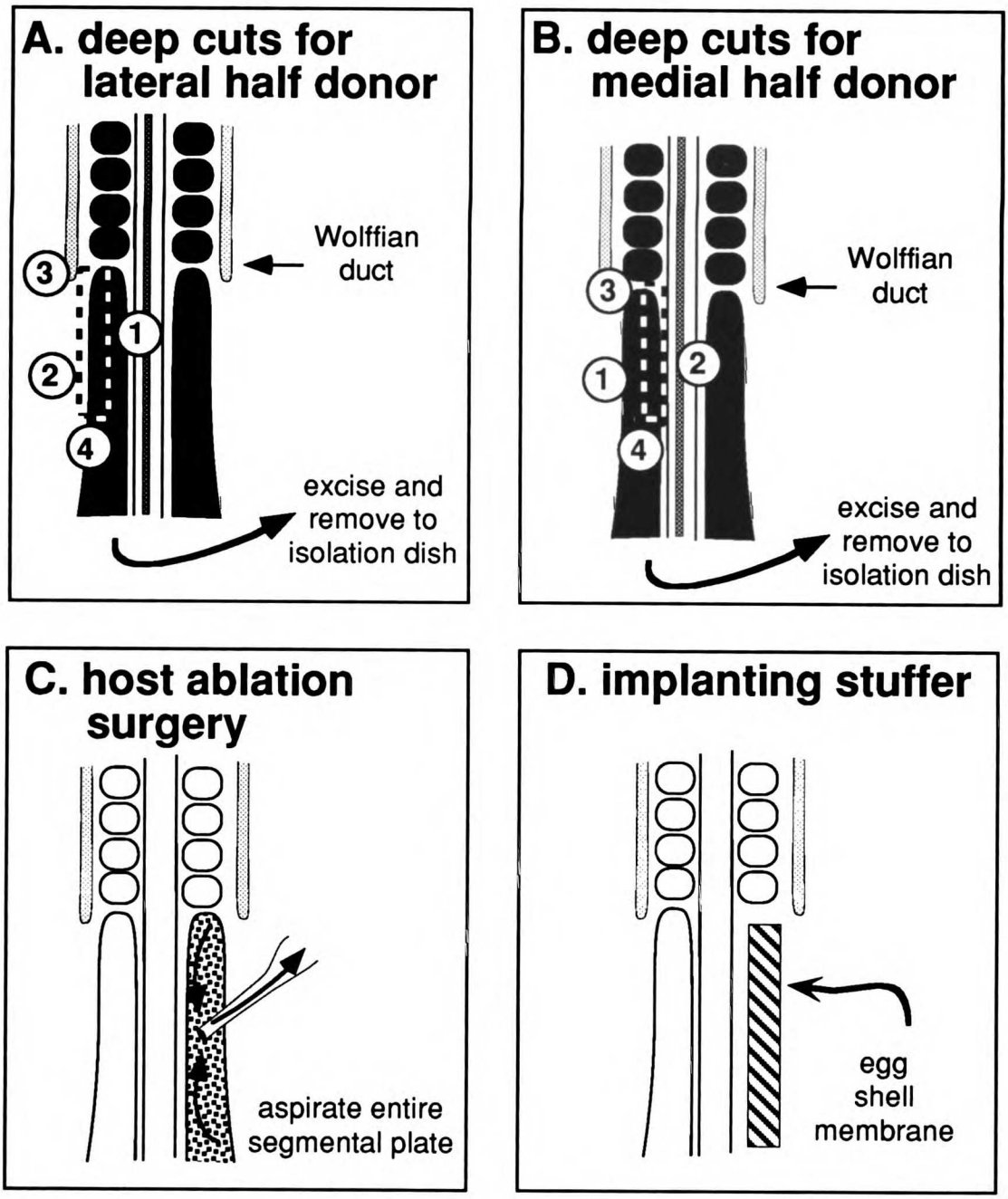
A. deep cuts for lateral half donor
Wolffian duct
3
1
2
4
excise and remove to isolation dish
B. deep cuts for medial half donor
Wolffian duct
3
1
2
4
excise and remove to isolation dish
C. host ablation surgery
aspirate entire segmental plate
D. implanting stuffer
egg shell membrane

Figure 1.3

Figure 1.4. 5 day old chimeric embryo after segmental plate graft.

(A) Cross section at the thoracic level of a chimeric embryo stained with the anti-quail antibody. The neural tube (N.T.), notochord (N.C.), coelom (arrow) and paraxial mesoderm are visible. Note that in contrast to the Feulgen technique, the antibody technique allows visualization of individual cells at low magnification.

(B) Adjacent cross section of the chimeric embryo shown in (A), stained with the anti-muscle myosin antibody, MF20. Skeletal muscle cells (arrow) are stained dark blue, and are formed from the grafted quail cells on the operated side of the embryo.

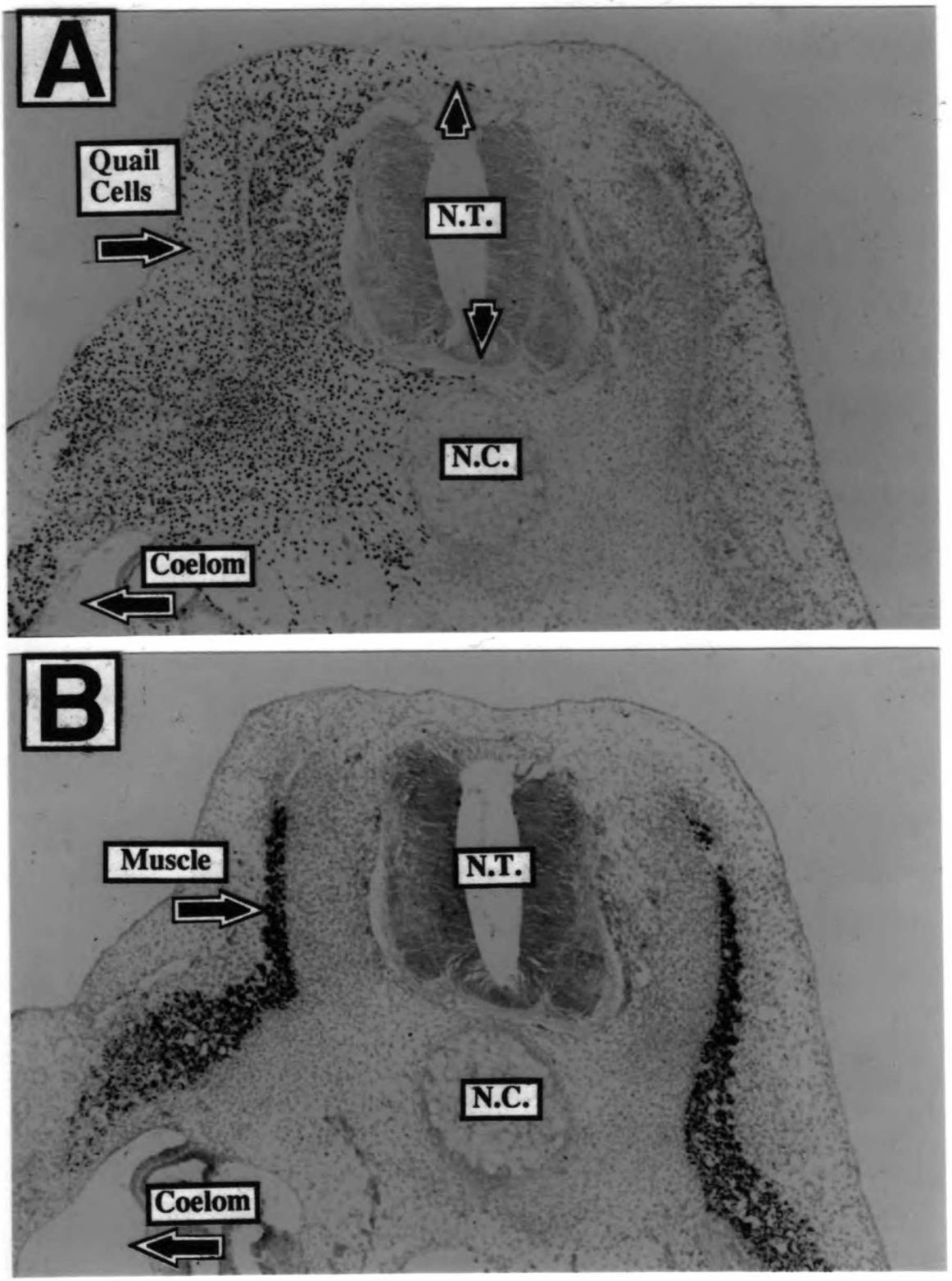

Figure 1.4

CHAPTER TWO

Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification

Summary

Specification of the myogenic lineage begins prior to gastrulation and culminates in the emergence of determined myogenic precursor cells from the somites. The myoD family (MDF) of transcriptional activators controls late step(s) in myogenic specification that are closely followed by terminal muscle differentiation. Genes expressed in myogenic specification at stages earlier than MDFs are unknown. The *Pax-3* gene is expressed in all the cells of the caudal segmental plate, the early mesoderm compartment that contains the precursors of skeletal muscle. As somites form from the segmental plate and mature, *Pax-3* expression is progressively modulated. Beginning at the time of segmentation, *Pax-3* becomes repressed in the ventral half of the somite, leaving *Pax-3* expression only in the dermomyotome. Subsequently, differential modulation of *Pax-3* expression levels delineates the medial and lateral halves of the dermomyotome, which contain precursors of axial (back) muscle and limb muscle, respectively. *Pax-3* expression is then repressed as dermomyotome-derived cells activate MDFs. Quail-chick chimera and ablation experiments confirmed that the migratory precursors of limb muscle continue to express *Pax-3* during migration. Since limb muscle precursors do not activate MDFs until 2 days after they leave the somite, *Pax-3* represents the first molecular marker for this migratory cell population. A null mutation of the mouse *Pax-3* gene, *Splotch*, produces major disruptions in early limb muscle development (Franz et al., 1993; Goulding et al., 1994). We conclude, therefore, that *Pax-3* gene expression in the paraxial mesoderm marks earlier stages in myogenic specification than MDFs and plays a crucial role in the specification and/or migration of limb myogenic precursors.

Introduction

All of the skeletal muscles of the avian body (excluding those of the head) arise from progenitor cells derived from the somites, condensations of paraxial (or segmental) mesoderm that form on either side of the developing neural tube (Wachtler and Christ, 1992). The medial and lateral halves of the somite are derived from a region of Hensen's node and a region of the primitive streak, respectively (Selleck and Stern, 1991) and give rise to at least two myogenic precursor populations at forelimb-levels (Ordahl and Le Douarin, 1992; Ordahl, 1993). Cells in the medial halves of newly-formed somites give rise to the embryonic myotome, the primordial muscles of the vertebral column. In contrast, cells in the lateral halves of newly formed somites migrate away from the somite region and enter the developing wing bud, where they form the muscles of the limb. Although these cell movement and fate aspects of myogenic development from somite precursors are becoming increasingly understood, the cellular and molecular mechanisms underlying myogenic specification in the embryonic somite are not well understood.

Myogenic determination factors (MDFs), are members of the myoD family of helix-loop-helix transcriptional activators that are exclusively expressed in cells of the skeletal muscle lineage (Ott, et al., 1991; Pownall and Emerson, 1992). MDFs are believed to play central roles in the transcriptional regulation of most, and possibly all, vertebrate skeletal muscle-specific genes (Olson, 1992; Ordahl, 1992; Emerson, 1993). More importantly, MDFs are also implicated in the process of specification of developing vertebrate skeletal muscle (Weintraub, et al., 1991). Forced expression of MDFs converts diverse non-myogenic cell types into cells of the myogenic lineage (Weintraub, et al., 1989; Choi, et al., 1990), whose differentiation programs are indistinguishable from those of primary cultures of myogenic cells isolated from embryos (Holtzer, et al., 1991). MDFs are also

the earliest known molecular markers that are expressed in the somitic precursor cells of the myotome, the primordia of the axial musculature and the first skeletal muscle to appear developmentally (Ott, et al., 1991; Pownall and Emerson, 1992).

The mechanism(s) by which MDFs control cell determination are unknown but other factors are likely to also be involved in myogenic specification. First, myogenically *competent* cells (embryonic cells capable of differentiating into muscle in a permissive environment) first appear during gastrulation and become sequestered in the segmental plate, many hours prior to MDF activation in the somites (Krenn, et al., 1988; Choi, et al., 1989). Therefore, important molecular changes in myogenic specification must precede MDF activation. Second, MDFs are not expressed in the lateral half of the somite (Ott, et al., 1991; Pownall and Emerson, 1992) which contains the migratory precursors of limb muscle (Christ, et al., 1974; Chevallier, et al., 1977; Christ, et al., 1977; Ordahl and Le Douarin, 1992). Activation of MDFs in these cells occurs immediately prior to terminal differentiation, within the limb bud, two days after they have left the somite (de la Brousse and Emerson, 1990; Ott, et al., 1991). Therefore, MDF expression is probably not a determination marker for these precursor cells because they are determined before MDFs are expressed (Bonner and Hauschka, 1974; Dienstman et al., 1974; White, et al., 1975; Kieny, 1980; Mauger and Kieny, 1980; Mauger et al., 1980; Rutz et al., 1982; Seed and Hauschka, 1984). Modulation of the determination of a minority of migratory cells may occur under extreme conditions (Kieny et al., 1981). Finally, transgenic mice homozygous for null alleles of myf-5 and myo-D, the earliest MDFs expressed in the mouse developmental sequence, show little or no muscle deficit (Braun, et al., 1992; Rudnicki, et al., 1992) although ablation of myogenin expression, an MDF expressed at the onset of terminal differentiation, has a drastic effect on muscle development (Harvey, 1990; Nabeshima, et al., 1993). Such observations indicate that additional factors must act prior

to, as well as concommitant with, MDFs to specify myogenic lineages during embryonic development.

In order to better understand the molecular basis for myogenic cell specification, we have sought markers for early somitic precursors of muscle, particularly those that migrate to the limb bud. Such markers would allow these cells to be identified and characterized, and may lead to the identification of other gene products that might interact with, or precede, MDFs during the process of myogenic specification.

Pax genes encode a family of vertebrate transcription factors that contain a paired box, a conserved motif found in several *Drosophila* pattern formation genes (Gruss and Walther, 1992). In early mouse embryos, *Pax-3* expression is highly patterned in the developing nervous system (Goulding et al., 1991) and is found in the somite dermomyotome and the limb bud (Goulding, et al., 1994). Migration of somitic precursors to the limb bud is thought to occur in mammalian embryos, (Milaire, 1976) but has not yet been experimentally demonstrated (Beddington and Martin, 1989). Recently, *Splotch,* a mutant allele of the murine *Pax-3* gene, (Epstein et al., 1991; Goulding et al., 1993a) was shown to produce major disruptions in limb muscle development (Franz, 1993; Franz, et al., 1993). Thus, *Pax-3* gene expression in the somite dermomyotome may be important in myogenic specification, particularly for migratory precursors to limb muscle.

Here we use in situ hybridization and embryo surgery to analyze the expression of the *Pax-3* gene during the development of the somites in avian embryos. We find that changes in *Pax-3* expression within the dermomyotome define specific myogenic precursor populations at stages preceding MDF activation and the onset of terminal differentiation.

Methods and Materials

Embryos

Fertile eggs of the white leghorn chicken (*Gallus domesticus*) and the japanese quail (*Coturnix coturnix japonica*) were obtained from Western Scientific Products (Sacramento, CA) and Strickland Farms (Pooler, GA.) respectively. Eggs were incubated at 37°C in a forced draft incubator and embryos staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951; Hamburger and Hamilton, 1992).

Somite Staging

Somites were staged according to a method whereby the most newly-formed somite is designated stage I, and progressively older (more rostral) somites designated by increasing roman numeral values (Ordahl, 1993). In sectioned embryos, inter-somitic boundaries were used to establish the stage of a particular somite in cross-section. The somites shown at stages IV (Fig. 1C-E) and XIII (Fig. 2A-D), are somites number 26 and 17, respectively, of the same 29-somite embryo.

In situ hybridization

Embryos were dissected from the area pellucida and pinned out in Sylgard coated dishes for fixation in 4% paraformaldehyde/PBS (pH 7.1) for 4-24 hours. Fixed embryos were dehydrated through an ethanol gradient and embedded in Paraplast for sectioning. Adjacent 5 mm transverse sections were processed for in situ hybridization as described elsewhere (Frohman et al., 1990), using the following [^{35}S]-labelled cRNA probes: (1) chick *Pax-3* probe, a 336 bp EcoRV/BamHI fragment (Goulding et al., 1993b) (2) chick myoD probe, a 622 bp PvuII/EcoRI fragment (Lin et al., 1989) and (3) quail myf-5 probe, an 850 bp EcoRI/XhoI fragment of cC528 (Pownall and Emerson, 1992). Sections were

exposed for 3-14 days and then developed and stained with hematoxylin and eosin. Hybridization patterns were visualized using either dark field optics or epi-illumination on a Zeiss Axiophot.

Embryonic surgery

Chicken eggs were windowed when the embryos had developed to stage 12 HH (Hamburger and Hamilton, 1951; Hamburger and Hamilton, 1992). Black ink (Pelican #16) was injected between the blastoderm and the yolk to improve visualization of embryonic tissues, and the vitelline membrane removed. Tungsten microscalpels, electrolytically sharpened in 0.5 M KOH, were used to cut through the ectoderm overlying the segmental plate on the right side of the embryo at the brachial level. A small amount of pancreatin (GIBCO 4X) was then introduced by mouth pipette to facilitate the reflection of a flap of ectoderm away from the segmental plate mesoderm. A length of rostral segmental plate equivalent to approximately 6-8 somites was then removed after cutting between it and the intermediate mesoderm laterally, followed by gentle separation from the neural tube medially, and the endoderm, ventrally. The segmental plate fragment was removed using a mouth pipette, and the excised area was rinsed at least three times with Tyrode's salt solution, and the ectoderm flap replaced. For extirpation experiments, the shell was re-sealed at this point with Scotch tape (3M) and returned to the incubator. Embryos were then incubated to stage 24 HH and survivors fixed and processed for in situ hybridization as described above (n=7). Serial sections were examined for evidence of *Pax-3* and myoD expression at rostral levels of the wing bud, where somites and somite-derived tissue were absent. At more caudal levels of the wing bud, somites were present and some *Pax-3* expressing cells were present in the adjacent wing bud, presumably due to expansion of caudal segmental plate cells into the extirpated region.

For chick/quail grafts, hosts were prepared as described above, and a matching piece of segmental plate mesoderm, removed using the same procedure from an equivalently staged quail embryo, was inserted in place of the host (chick) segmental plate. After replacing the host ectodermal flap over the grafted segmental plate, the eggs were re-sealed and incubated to stage 24 HH, at which time surviving embryos (n = 11) were fixed in Carnoy's fluid and processed for Feulgen staining as described (Le Douarin, 1973) and in situ hybridization as described above.

Results

Pax-3 expression in early somite development.

The paraxial (or segmental) mesoderm is comprised of the segmental plate, a narrow sheet of mesoderm on either side of the neural tube, and the somites, epithelial balls that bud off at the rostral end of the segmental plate at regular intervals. Once formed, epithelial somites undergo a series of cellular movements and transformations leading to the development of: (i) the sclerotome, which is the precursor of the axial skeleton, (ii) the myotome, which is the back (axial) muscle primordium, and (iii) at limb levels, migratory cells that give rise to the muscles of the limbs (Christ, et al., 1974; Chevallier, et al., 1977; Christ, et al., 1977; Beresford, 1983). At much later stages, another group of mesenchyme cells leaves the somite to give rise to the dermis of the back. Since somites arise sequentially during early development, the somite ladder of an embryo represents a caudo-rostral array of progressively later stages of somite development (Ordahl, 1993). These stages are represented by roman numerals, with stage I representing the most newly formed somite, stage II somite the next oldest, and so forth. We compared the expression of *Pax-3* in developing somites with that of myoD and/or myf-5.

Figures 2.1 and 2.2 show the *Pax-3* expression patterns in the segmental mesoderm of a stage 17 HH embryo, a stage of chick development at which myotome formation and cell migration is underway in wing-level somites. *Pax-3* is expressed homogeneously at all axial levels of the segmental plate (Fig. 2.1A) except at the extreme rostral end, where segmental plate cells condense to form new somites. At this location (Fig. 2.1B), *Pax-3* expression becomes localized dorsally, and spans the medio/lateral limits of the dorsal epithelium of the somite, the future dermomyotome. The ventral epithelium and core of the somite, which do not detectably express *Pax-3*, are destined to dissociate into mesenchyme at subsequent stages of somitogenesis.

By somite stage IV, *Pax-3* expression is higher in the lateral half of the dermomyotome as compared to the medial half (Fig. 2.1E). *Pax-3* expression is undetectable, however, in the medial lip of the dermomyotome where expression of myoD (the first MDF expressed in avian somites) has been activated (Fig. 2.1D). Thus, at early stages of somitogenesis, expression of the *Pax-3* gene is repressed in cells that have activated myoD expression, and modulated to different levels in the lateral and medial portions of the dermomyotome (summarized in Fig. 2.1F).

Pax-3 expression during myotome formation.

Myotome formation begins at stage VII of somite development, as cells from the dermomyotome begin to form a second layer of differentiated, post-mitotic muscle cells beneath the dermomyotome epithelium (Kaehn, et al., 1988). A later stage of this process is depicted in Figure 2.2. Panel A shows *Pax-3* hybridization in the dermomyotome of a stage XIII somite, where the boundary between high- and low-level *Pax-3* expression is now located about one-quarter of the distance from the lateral edge (white arrow). The adjacent section hybridized to myf-5 (Panel B) shows that myotome formation has progressed as far as the medial edge of the somite cavity. The strict segregation of *Pax-3*

hybridization to the precursor cells of the medial portion of the dermomyotome (dm), and myf-5 hybridization to the differentiated cells of the myotome proper (m), is evident at high magnification (Panels C and D, respectively). Thus, the non-overlap between *Pax-3*-positive and myf-5-positive cells extends to all regions of the somite at these early stages of somitogenesis.

Pax-3 expression in migratory limb muscle precursors and at later stages of somitogenesis

In early limb bud stage embryos (stage 18 HH), *Pax-3* expression extends in a spatially continuous domain from the dermomyotome to the limb bud (Fig. 2.3A), suggesting it marks myogenic precursors migrating from the lateral edge of the dermomyotome. As expected, expression of myoD and myf-5 is not detectable in the limb bud at this stage (Figs 2.3B, C). By stage 21 HH, *Pax-3* expression in the limb bud has segregated into dorsal and ventral domains (Fig. 2.3D) that prefigure the dorsal and ventral limb muscle masses that begin to differentiate at stage 24 HH (see Figs 2.3G, H). These domains do not express either myoD or myf-5 at stage 21 HH (Figs 2.3E, F).
By stage 24HH, myoD expression is initiated in the limb bud within the *Pax-3* expression domains (Figs 2.3G, H).

Figure 2.3 also shows that at these later stages of somitogenesis, *Pax-3* expression intensifies in the dermomyotome epithelium and also becomes progressively dispersed over the myotome. The progressive overlap of *Pax-3* hybridization grains over the myoD- and myf-5 positive myotome at stages 18 and 21 HH (Fig. 2.3A-C and D-F, respectively) may represent the migration of dermomyotome precursors of secondary muscle fibers into the myotome (B. Christ, personal communication).

Experimental demonstration that *Pax-3* marks migratory precursors of limb muscle

Pax-3 expression domains in the limb could represent myogenic precursors migrating from the somite dermomyotome, or alternatively, de novo *Pax-3* gene activity in somatic mesoderm cells derived from limb bud mesenchyme. To distinguish between these possibilities, two types of embryo surgery experiments were performed (Fig. 2.4).

In the first series of experiments (Fig. 2.4, experiment #1), the segmental plate was extirpated on the right sides of stage 12 chick embryos; the unoperated sides served as controls. After developing to stage 24, wing bud outgrowth in these embryos was normal (Fig. 2.3I), as expected, because outgrowth is principally due to proliferation of limb bud mesenchyme (Chevallier et al., 1978). However, both the somite region and corresponding regions of the limb bud were devoid of detectable *Pax-3* signal (Fig. 2.3I) or myoD signal (not shown). The absence of *Pax-3* expression in the developing wing bud, therefore, demonstrates that *Pax-3* expression domains in the wing bud depend upon the presence of the adjacent somites.

The *Pax-3* hybridization pattern of these embryos was normal on the control side, and interestingly, normal in the neural tube on the operated side (Fig. 2.3I and data not shown). Thus, *Pax-3* localization in the neural tube is not dependent upon the adjacent paraxial mesoderm.

In the second series of experiments, somite derived cells were positively identified in the limb bud and their *Pax-3* expression demonstrated in adjacent sections by in situ hybridization. Wing-level segmental plates were replaced in stage 12 HH chick embryos with those of quail (Fig. 2.4, experiment #2). After development to stage 24 HH, somite-derived quail cells in the limb dorsal and ventral muscle masses were identified by their distinctive nucleolar marker (Le Douarin, 1973). Adjacent sections were hybridized to the *Pax-3* probe, and the *Pax-3* expression domains (Fig. 2.5B, D, F, H, J) were found to align with the quail cell domains in the corresponding Feulgen-stained sections (Fig. 2.5A,

C, E, G, I). Two types of boundaries between somite-derived quail cells and limb mesenchyme cells were examined in detail.

First, the region immediately beneath the apical ectodermal ridge of the developing limb bud represents a growth zone from which migratory muscle precursors are excluded (Newman et al., 1981; Rutz, et al., 1982). This region is progressively magnified in panels A, C and E of Figure 5. In Panel E, the boundary between somite-derived quail cells and limb mesenchyme chick cells can be seen (exemplar quail cell nuclei indicated by black arrows, chick cell nuclei by white arrows). Corresponding panels B, D and F show that the distal limit of *Pax-3* expression corresponds closely to that of the invasive myogenic precursor cells of quail origin.

A second type of segregation boundary occurs between limb mesenchyme and somite-derived cells as they condense into the central pre-chondrogenic mass, and the dorsal and ventral pre-muscle masses, respectively (Searls and Janners, 1969; Bonner and Hauschka, 1974; Stark and Searls, 1974; Mauger and Kieny, 1980). This segregation boundary is progressively magnified in Figure 2.5, panels A, G and I (quail cells indicated by black arrows, chick cells by white arrows) with the corresponding *Pax-3* hybridization shown in panels B, H and J. In this region the *Pax-3* hybridization signal is again closely correlated with the presence of somite-derived quail cells, indicating that the segregation of chondrogenic and myogenic precursor cells is
reflected by the *Pax-3* hybridization signal which is restricted to the pre-myogenic domain.

This segregation of *Pax-3* hybridization to regions populated by quail cells was seen in all boundary regions of the limb bud and indicates that the somite-derived myogenic precursor cells continue to express *Pax-3* after leaving the dermomyotome epithelium. The possibility that the small proportion of wing mesenchyme cells that also reside in the pre-muscle masses are secondarily induced to express *Pax-3*, cannot be ruled out at this time. Although unlikely, such induction in wing mesenchyme-derived cells would be potentially

interesting because it is well established that such cells are non-myogenic (Mauger, et al., 1980). By day 8 of development, all *Pax-3* expression is absent in the limb and somite region. *Pax-3* hybridization was never observed in the dermis, another cell population that arises from the dermomyotome (data not shown).

Discussion

Pax-3 as a marker for migratory myogenic precursors.

An important finding from this study is that the migratory dermomyotome precursors of limb bud muscle express *Pax-3*. No other molecular marker exists for these cells. These precursors are derived from the lateral half of the dermomyotome, which expresses *Pax-3* at higher levels than the medial half of the dermomyotome. Cells migrating to the limb bud also express *Pax-3* at similar levels. Since *Pax-3* expression appears to be homogeneous in the lateral region of the dermomyotome, we hypothesize that the migratory *Pax-3*-positive cells are derived from the *Pax-3*- positive cells in the lateral dermomyotome. The onset of terminal myogenic differentiation in the limb bud, as detected by MDF expression, occurs within the domain of *Pax-3* expressing cells, suggesting that differentiated myocytes are recruited from the *Pax-3* positive cells (see below). Using embryo surgery and the quail-chick marking system, we were able to conclusively demonstrate that it is somite-derived precursor cells that express *Pax-3* within the developing limb bud.

The demonstration of *Pax-3* as a marker for migratory limb muscle precursors in avian embryos may also be important for the understanding of mammalian limb muscle development. Although it is widely assumed that limb muscle precursors in mammals arise from migratory somite cells (Milaire, 1976) such migration has not been experimentally demonstrated (Beddington and Martin, 1989). However, *Pax-3* expression domains in

mouse embryo limb buds are similar to those seen here for avian embryos and, more importantly, are absent in the limbs of *Splotch* embryos (Goulding, et al., 1994). Therefore, *Pax-3* expression is likely to also mark mammalian migratory precursors of limb muscle. The experimental characterization of the *Pax-3* marker for these cells in avians, via somite ablation and quail-chick transplantation, now provides a basis for more detailed molecular analysis of these precursors, and/or a reference marker for the discovery of other markers co-expressed in these migratory cells in both the avian and mammalian systems.

Pax-3 expression marks definable compartments of the dermomyotome.

Figure 2.6 summarizes the expression of *Pax-3* in the paraxial mesoderm. As the somite develops, *Pax-3* gene expression becomes restricted to the dermomyotome. Subsequent changes in *Pax-3* expression delineate compartments of the dermomyotome, and its derivatives, that are definable by other criteria (see Figure 2.6). At somite stage IV, at least 3 such compartments are identifiable (Fig. 2.6D): (1) the lateral half of the dermomyotome, which contains myogenic precursors that will migrate to the limb, expresses *Pax-3* at high relative levels, (2) the medial half of the dermomyotome, which contains precursors of the myotome and dermis, expresses *Pax-3* at relatively low levels, (3) the medial lip, which has activated the myoD gene, and has repressed *Pax-3* expression to an undetectable level.

By somite stage X, two additional compartments of dermomyotome derivatives are evident (Fig. 2.6E): (4) myogenic precursor cells migrating from the lateral half of the dermomyotome on their way to the limb bud that express *Pax-3* at relatively high levels, (5) differentiated myotome cells that are derived from the medial half of the dermomyotome and have now ceased *Pax-3* expression and activated MDFs and contractile protein genes. Thus, *Pax-3* marks dermomyotome cells at early stages of specification, prior to MDF activation that presages myogenic terminal differentiation.

At late stages of development, MDF and *Pax-3* expression domains increasingly overlap. We interpret (Fig. 2.6F) this overlap in the limb bud as recruitment of MDF-positive/*Pax-3* negative, differentiating myocytes from the *Pax-3*-positive/MDF-negative precursor population. Similarly, in the back muscle region, *Pax-3* positive cells from the dissociating dermomyotome are invading the myotome to act as precursors of secondary muscle fibers. The alternative hypothesis, that the *Pax-3* gene is being re-activated in some differentiating myocytes, cannot be ruled out at this time. *Pax-3* expression was not detected in dermal cells, a late derivative of the dermomyotome, suggesting that *Pax-3* expression may also be downregulated as dermomyotome cells differentiate along this pathway.

Pax-3 gene expression and myogenic specification.

The correlation between *Pax-3* modulation and the dermomyotome compartments described above indicate that *Pax-3* gene expression is a marker for early steps in the specification of dermomyotome cells. *Pax-3* gene modulation may also mark much earlier steps in the specification of paraxial mesoderm.

An important event in paraxial mesoderm specification is the acquisition of competence to form skeletal muscle, a property unique to the paraxial mesoderm (Krenn, et al., 1988). Cells with such competence first appear in the primitive streak and Hensen's node and subsequently segregate to the segmental plate and somites (Krenn, et al., 1988). *Pax-3* expression shows a similar distribution in mesoderm, first detectable in cells leaving the primitive streak and Hensen's node (Goulding, et al., 1993b) and then becoming localized to the segmental plate and somites (this manuscript and (Goulding, et al., 1994).

A second major step in paraxial mesoderm specification occurs at the time of segmentation, when the rostro-caudal and dorso-ventral axes of the somite are thought to be established (Keynes and Stern, 1988). We show here that *Pax-3* expression in the

segmental plate is dorsalized at this point. This dorsalization is under control of the notochord (Goulding, et al., 1993b), which is known to influence the specification of somite cells (Halpern et al., 1993; Pourquie, et al., 1993). The rostral segmental plate is also the site where paraxial mesoderm up-regulates *Motch* (Reaume et al., 1992) the mouse homologue of *Notch*, a *Drosophila* gene involved in the specification of cell types derived from embryonic epithelial structures (Hartenstein et al., 1992). *Notch* appears to influence the specification of *Drosophila* muscle cells (Corbin, et al., 1991), as well as the binary decision between neural and epidermal cell fates (Heitzler and Simpson, 1991).

The *Pax-3* gene appears to be repressed when dermomoyotome-derived cells activate expression of the MDFs, suggesting that *Pax-3* gene expression in dermomyotome precursor cells may be negatively coupled to activation of the myogenic gene cascade. We note in this regard that the MHox gene, which is highly expressed in skeletal muscle, and whose product binds to muscle-specific enhancer sequences, contains a paired-type homeodomain that is similar to that found in *Pax-3* (Cserjesi et al., 1992). It should be interesting, therefore, to examine the possible regulatory relationships between the *Pax-3* gene and other regulatory genes expressed in the muscle lineage, such as MDFs.

These observations indicate that *Pax-3* gene expression: (1) marks paraxial mesoderm cells from the time they gastrulate, (2) is modulated coincident with specification events occuring at the time of segmentation, (3) is again modulated in specific compartments of the dermomyotome, and (4) ultimately ceases as the dermomyotome precursors enter defined differentiated compartments such as skeletal muscle, and possibly dermis. We conclude, therefore, that modulation of *Pax-3* gene expression correlates with changes that occur during a broad window in paraxial mesoderm specification.

Pax-3 plays a direct role in specification of migratory limb muscle precursors.

Splotch, a mouse mutation that has been recently shown to affect muscle development (Franz, 1993; Franz, et al., 1993) is a mutant allele of the *Pax-3* gene (Epstein, et al., 1991; Goulding, et al., 1993a). *Splotch* mutant embryos show a profound deficit in limb muscles, with lesser effects on axial and body wall muscle. The fact that limb musculature, but not axial musculature, is affected in *Splotch* suggests that the *Pax-3* gene might play a differential role in the response of these two precursor populations to the extrinsic signals required for their differentiation. The development of myotomal muscle is sensitive to axial signals that do not affect development of limb muscle (Rong et al., 1992; Pourquie, et al., 1993). Similarly, migration of myogenic precursors from the somite is under control of the adjacent limb bud which does not affect the development of myotomal muscle (Chevallier, et al., 1977; Christ, et al., 1977). Thus, *Pax-3* activity might be required for the response of limb muscle precursors to signals from the limb bud.

The *Pax-3*-dependent response of dermomyotome cells to limb bud signals may be migration itself. *Pax-3* expressing cells are absent from the limb buds of *Splotch* embryos (Goulding, et al., 1994). The *Splotch* mutation is known to delay neural crest cell migration from the neural tube, (Moase and Trasler, 1990) and could similarly affect migration of dermomyotome cells. The cell adhesion molecule, N-CAM, is abnormally elevated in somites of *Splotch* embryos (Moase and Trasler, 1991) which could result in diminished capacity of dermomyotome cells to dissociate from the epithelium as a prerequisite to migration. Cytotactin is also expressed in the dermomyotome (Tan et al., 1991) and may initiate epithelial-mesenchymal transitions (Chiquet-Ehrismann, 1991). It is not known if either molecule is regulated by *Pax-3*, but N-CAM gene promoters are known to be regulated by other homeobox genes (Jones et al., 1992a; Jones et al., 1992b).

Finally, it is important to point out that *Pax-3* may not mark all migratory muscle precursors. Previous work indicates that the lateral half of wing-level somites gives rise to

limb musculature (Ordahl and Le Douarin, 1992). This implies that the *Pax-3*-negative cells in the ventro-lateral quadrant of the newly-formed somite are also precursors to limb muscle. Preliminary experiments indicate that these ventro-lateral somite cells migrate along a different route than the muscle precursors from the dermomyotome and may give rise to shoulder muscles (CPO, in preparation). Since the *Splotch* mutation also affects shoulder muscle development (Franz, 1993; Franz, et al., 1993), it will be interesting to determine whether this defect results from deficiency of *Pax-3* expression in the segmental plate, when these cells transiently express *Pax-3* at a stage prior to migration, or to other as yet unknown regulatory events.

Acknowledgements

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Figure 2.1. *Pax-3* expression in early somitogenesis.

Dark field visualization of *Pax-3* (A, B, E) and myoD (D) in situ hybridization in transverse sections taken at different axial levels of a stage 17 HH embryo (220X).

A. Mid-segmental plate. Uniform *Pax-3* expression is seen throughout the dorso/ventral and medio/lateral cross section of the segmental plate.

B. Rostral end of the segmental plate. *Pax-3* expression is localized to the dorsal epithelium uniformly from medial to lateral.

C-E. Adjacent cross sections through a stage IV somite (Ordahl, 1993) stained with hematoxylin and eosin (C), or hybridized to myoD (D) or *Pax-3* (E). *Pax-3* expression is reduced in the medial portion of the dermomyotome as compared to the lateral. *Pax-3* expression is undetectable in the medial lip of the dermomyotome where myo-D is expressed (Arrows).

F. Schematic summary of panels C-E, showing *Pax-3* expressed at high levels in the lateral half of the dermomyotome (black shading), at reduced levels in the medial half of the dermomyotome (grey shading), and at undetectable levels in the medial lip of the dermomyotome where myoD is expressed (striped shading).

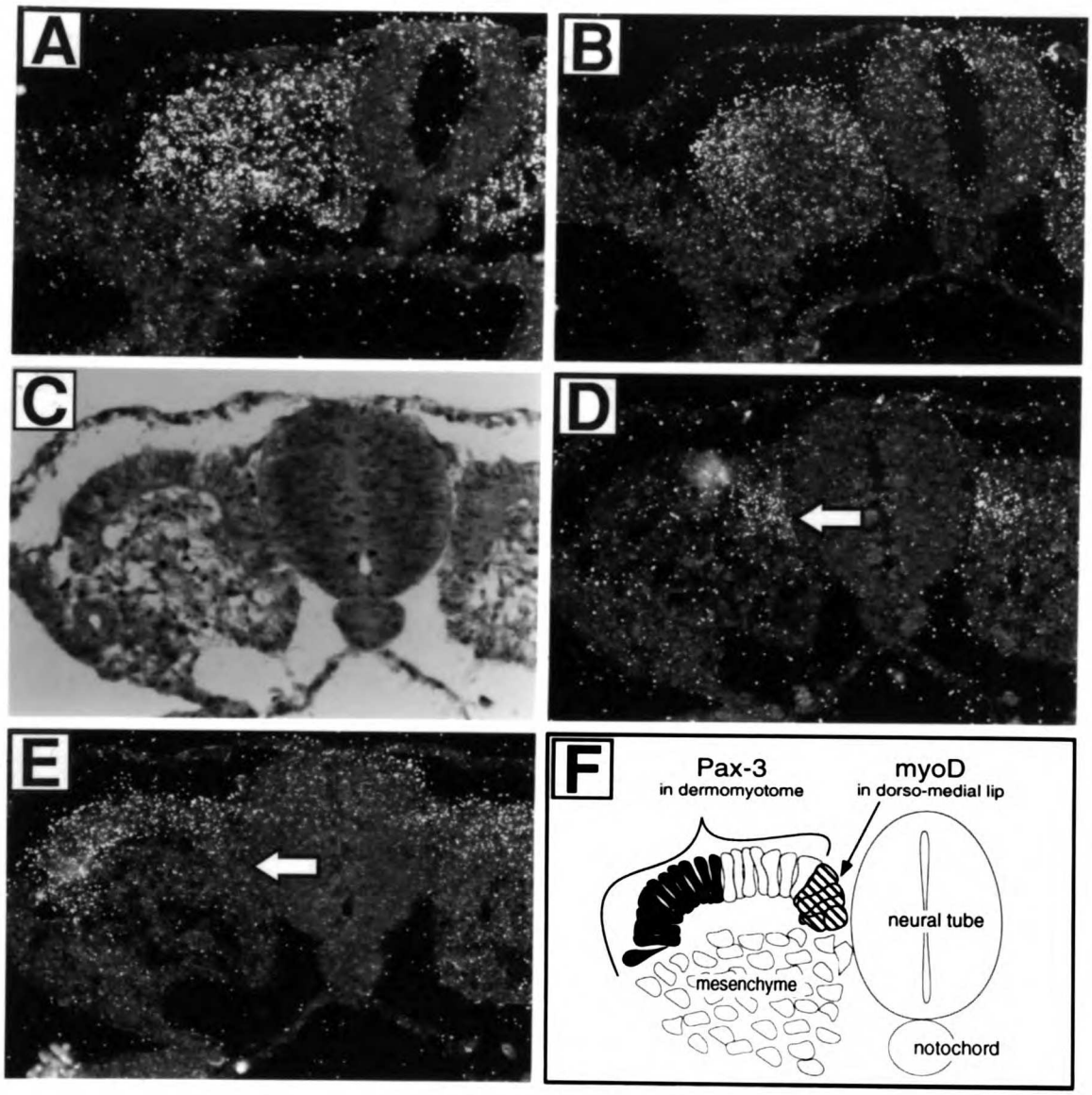
A
B
C
D
E
F
Pax-3
in dermomyotome
myoD
in dorso-medial lip
neural tube
mesenchyme
notochord

Figure 2.1

Figure 2.2. Anatomical separation of *Pax-3* and myf-5 expression.

Dark field (panels A, B) and epi-illumination (C, D) of limb-level somites hybridized to the *Pax-3* (A, C) and myf-5 (B, D) probes, respectively.

Panels A and B show a stage XIII wing-level somite at low magnification (220X). The white arrow indicates the approximate margin, at the lateral one third of the dermomyotome, that demarcates the high-expression and low expression domains of *Pax-3*.

Panels C and D show the myotome at higher (560X) magnification. The black arrow indicates the region where the somite cavity intervenes between the dermomyotome (dm) and myotome (m). Neural tube (nt), notochord (nc), sclerotome (sc), somite cavity (*).

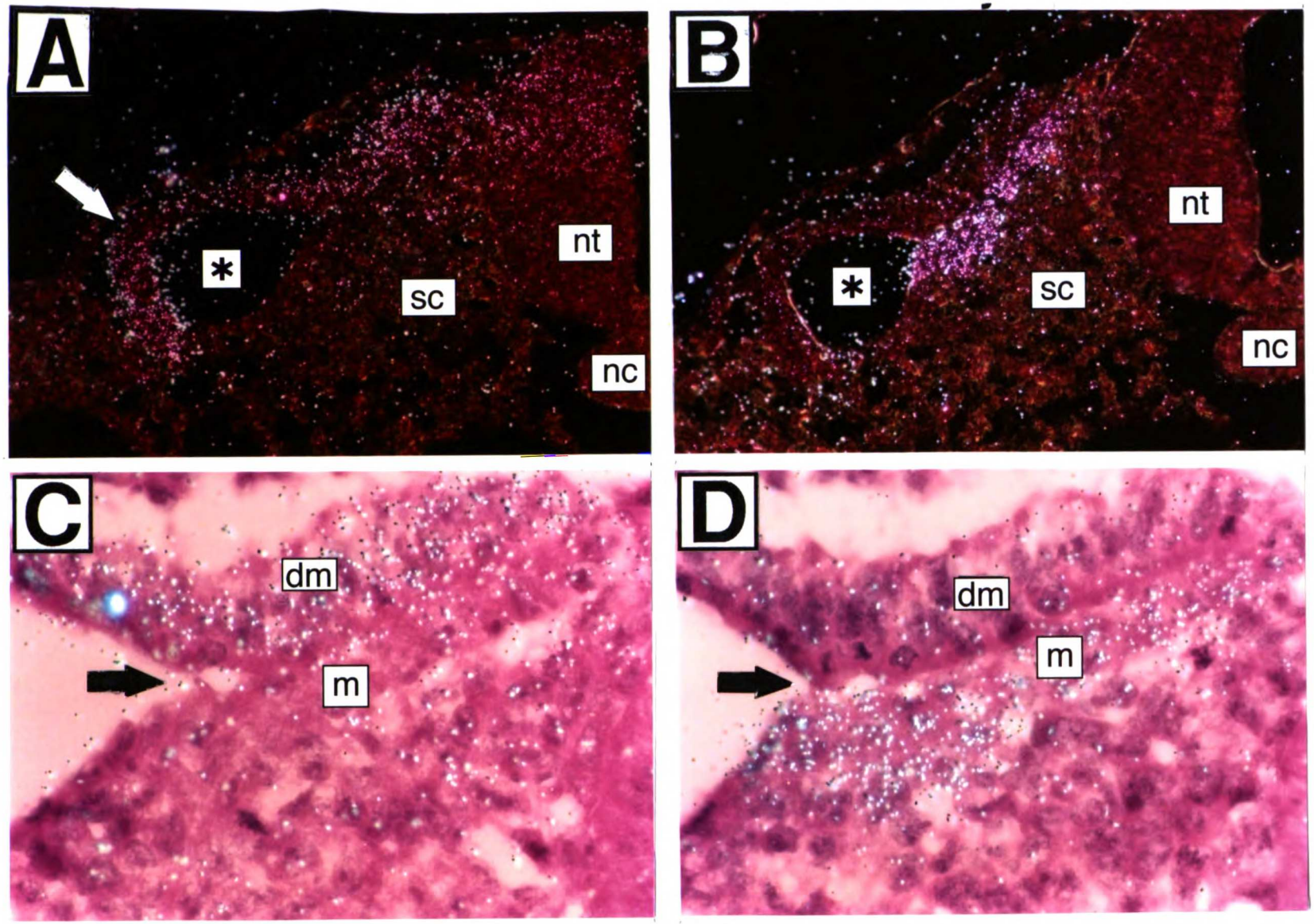

Figure 2.2

Figure 2.3. *Pax-3* expression in late somitogenesis and in the developing limb bud.

Panels A-H. Comparison of *Pax-3* hybridization in the limb bud with that of myoD and myf-5.
Adjacent sections of stage 18 HH (A-C; 110X), stage 21 HH (D-F; 56X) and stage 24 HH (G, H; 56X) embryos were hybridized with probes for *Pax-3* (A, D, G), myoD (B, E, H), and myf-5 (C, F).

Pax-3 hybridization in the dermomyotome (dm) extends in a continuous domain from the somite region into the limb bud (lb). At stage 18 HH, *Pax-3* expressing cells in the limb bud are widespread (arrowhead in A) but by stage 21 HH, these cells have coalesced into dorsal and ventral masses (arrowheads in D). Neither myoD nor myf-5 are detectable in the limb bud at either of these stages (arrowheads in B, C and E, F, respectively). By stage 24 HH, myoD expression (Panel H) appears within the dorsal and ventral *Pax-3* expression domains (Panel G) in the limb bud. Double arrows in G indicate proximal and distal limits of myoD expression within the *Pax-3* expression domains of the limb. Note that *Pax-3* expression in the dermomyotome (dm) begins to overlap the myoD- and myf-5-positive myotome (m) in the somite region (Panels D-F). *Pax-3* expression is also detected in the dorsal portion of the neural tube and portions of the developing spinal nerve.

Panel I: Ablation of paraxial mesoderm abolishes *Pax-3* expression in the limb bud (56X). Note altered orientation of this specimen. The segmental plate was removed from a stage 12 HH chick embryo which was then allowed to develop to stage 24 (Figure 4, experiment #1; and see Methods, for details). Ablated regions of the embryo were then sectioned and hybridized to probes for *Pax-3* expression (I) and myoD (not shown). Arrow and arrowheads in Panel I indicate expected position of dermomyotome and limb pre-muscle mass regions, respectively. *Pax-3* expression is normal on the unoperated side, but is not detectable on the operated side, indicating that expression in the limb required adjacent somitic mesoderm. Note that *Pax-3* expression in the neural tube is normal on the experimental side indicating that this expression pattern is independent of the paraxial mesoderm. MyoD expression was also absent in the limb and somite region on the operated side (not shown).

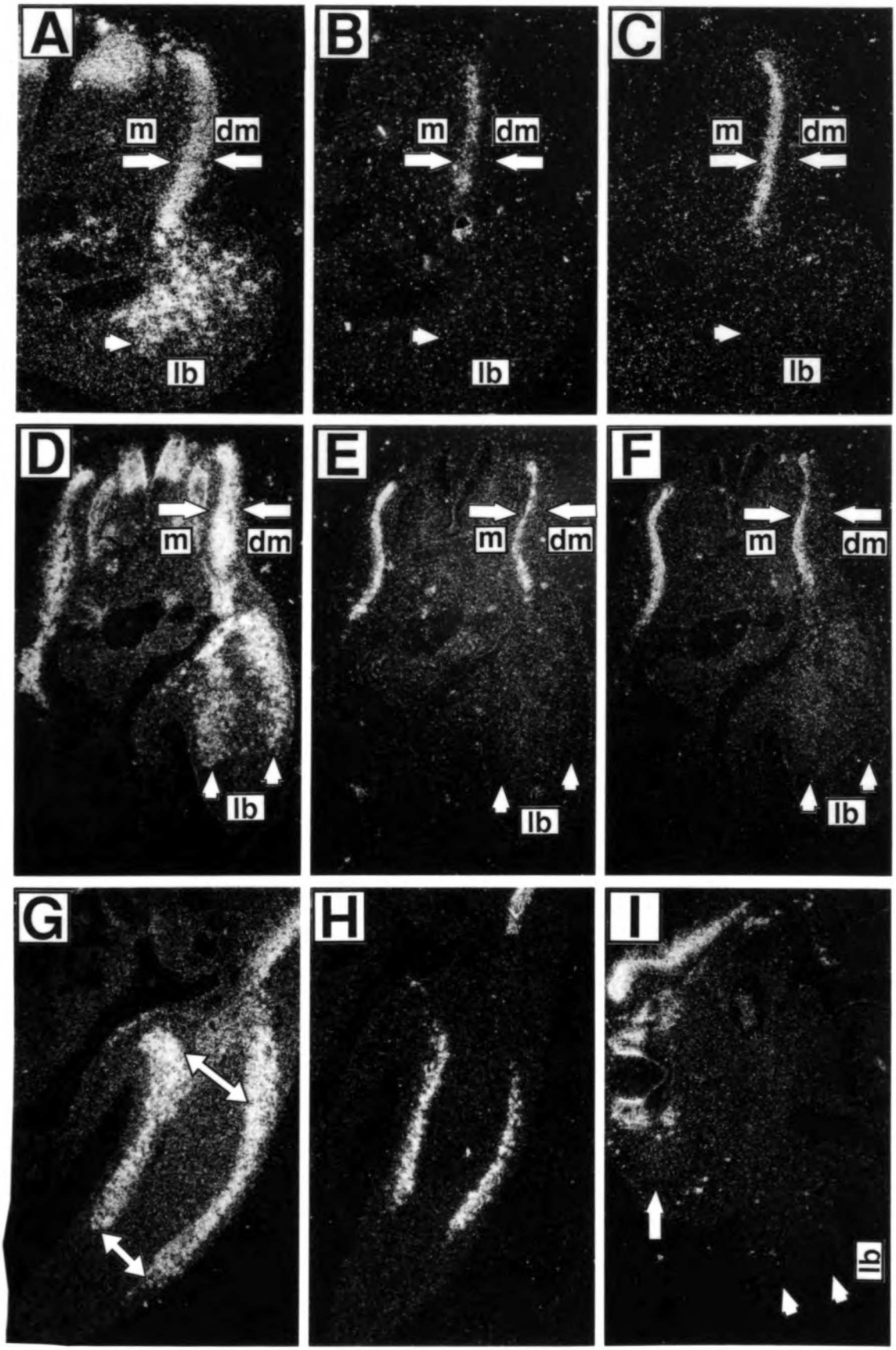

Figure 2.3

Figure 2.4. Diagrams of embryo surgery experiments.

Experiment #1. The segmental plate was removed from day 2 (stage 12 HH) chick embryos in ovo. After re-incubating for two days (to stage 24 HH), somitic derivatives are absent at the ablated levels, including sclerotome (s), myotome (m) and limb muscle. The outgrowth and morphogenesis of the limb mesenchyme, which is not derived from the somite, is unaffected by the absence of somite-derived muscle precursors.

Experiment #2. The segmental plate was removed from day 2 (stage 12 HH) chick embryos and replaced by the segmental plate of a stage 12 HH quail embryo. After re-incubating for two days (to stage 24 HH) the somitic derivatives of the chimeric embryo are composed of quail cells, including sclerotome (s), myotome (m) and limb muscle. By stage 24 HH, the somite-derived muscle and muscle precursors have coalesced into dorsal and ventral muscle masses, surrounded by limb mesenchyme.

Embryo surgery experiments

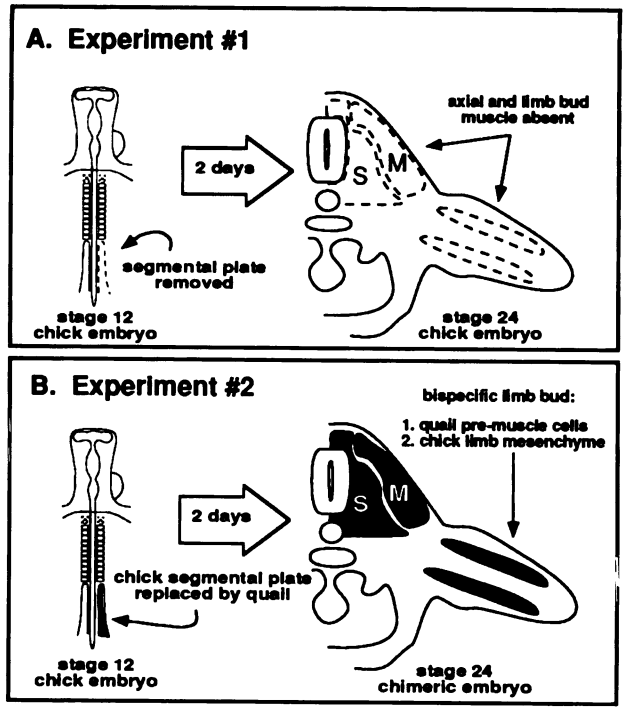

Figure 2.4

Figure 2.5. *Pax-3* expressing cells in the developing limb bud are somite-derived.

The segmental plates of stage 12 HH chick embryos were removed and replaced with those of quail (see Figure 4, experiment #2 and Methods, for details). After development to stage 24 HH, these chimeric embryos were sectioned and Feulgen stained to detect the quail nucleolar marker (A, C, E, G, I) and adjacent sections hybridized with the *Pax-3* probe (Panels B, D, F, H, J). Hybridization grains are visualized by dark field illumination in Panels B, D and H and by epifluorescence in Panels F and J.

Panels A and B: Low power (27X) views of Feulgen- and *Pax-3*-stained sections, respectively with boxed regions magnified in subsequent panels as indicated.

Panels C and D: Intermediate magnification (220X) of distal region of limb bud, beneath the apical ectodermal ridge (aer), where migratory somite cells have not yet invaded the limb bud mesenchyme (see text). Boxes show regions magnified in Panels E and F.

Panels E and F: Panel E is a magnified view (560X) view of the region boxed in C, showing the boundary between somite-derived quail cells with the distinctive dark purple nucleolar marker (black arrows) and chick limb mesenchyme cells with pale nucleoplasm (white arrows). The corresponding region of the adjacent section hybridized to the *Pax-3* probe is shown in Panel F. Note that at this boundary *Pax-3* hybridization grains co-localize with the quail cells and that the chick cell region contains background grain levels.

Panel G and H: Intermediate magnification (220X) of central region of the limb bud where somite- and limb mesenchyme-derived cells previously intermingled (see Fig. 2A) but have now segregated into: (i) a central chondrogenic region composed of limb mesenchyme-derived cells; and (ii) peripherally-localized pre-muscle masses of somite-derived myogenic precursors (see text). Boxes show regions magnified in Panels I and J.

Panels I and J: Panel I is a magnified view (560X) of the region boxed in G, showing the boundary between the core-region chick limb mesenchyme cells (white arrows) and the adjacent pre-muscle mass containing somite-derived quail cells (black arrows). The corresponding region of the adjacent section hybridized to the *Pax-3* probe is shown in Panel J. Note that the segregation of the *Pax-3 h*ybridization grains corresponds to the pre-muscle region containing quail cells and that the chondrogenic core region, containing chick cells, shows only background levels of hybridization.

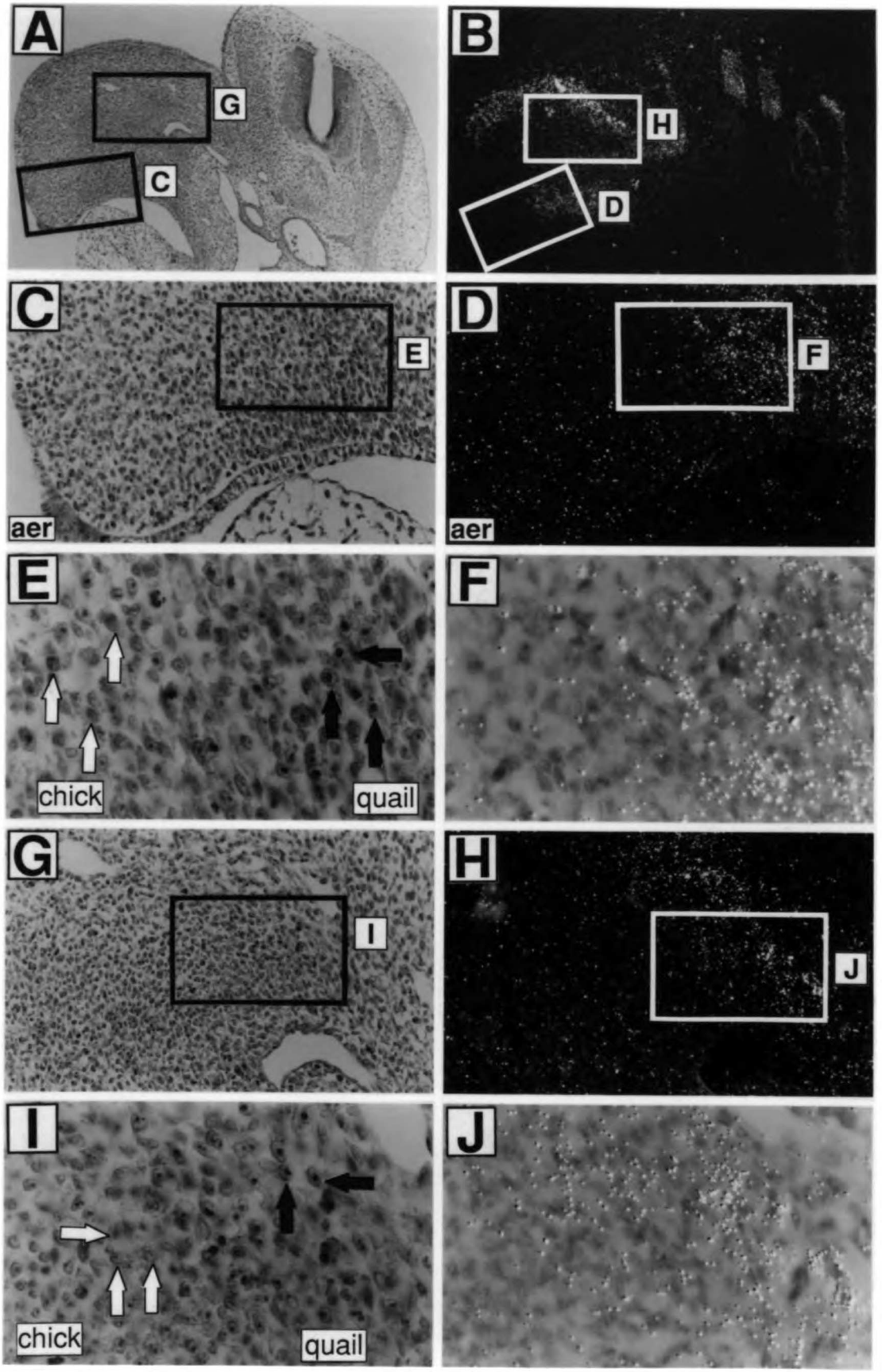

Figure 2.5

Figure 2.6. Generalized summary of *Pax-3* expression in somitogenesis.

Panel A: Diagrammatic representation of a stage 15 HH embryo containing 25 formed somites. Lines marked B-E indicate the levels at which transverse views of this embryo are presented in subsequent panels.

Panel B: Transverse section through the mid-segmental plate region. *Pax-3* expression (black shading) is homogeneous in the segmental plate at this level.

Panel C: In a stage I somite (most recently formed somite) the cells have become organized into an epithelial ball. *Pax-3* expression is localized to the dorsal half of the somite at this stage and is expressed at equivalent levels in the dorso-medial and dorso-lateral epithelium.

Panel D: In a stage IV somite (4th most recently formed somite) the ventral (*Pax-3*-negative) region of the somite has formed mesenchyme while the dorsal region has remained epithelial as the dermomyotome. *Pax-3* expression is higher in the lateral half of this epithelium (1, black shading) than in the medial half (2, grey shading). In the medial lip of the dermomyotome (3), myoD expression has been activated while *Pax-3* expression is repressed (stippled shading).

Panel E: In a stage X somite at least 5 distinct populations of dermomyotome-derived cells can be defined by *Pax-3* expression, anatomical position and/or developmental fate:
The three compartments first identified in Panel D still remain in a stage X somite: (1) Lateral dermomyotome cells are still epithelial but are destined to migrate to the limb bud as myogenic precursor cells. These cells are strongly *Pax-3*-positive (black shading), and negative for myoD and myf-5. (2) Medial dermomyotome cells are weakly *Pax-3*-positive, and negative for myoD and myf-5. (3) Medial lip dermomyotome cells that form a ridge of myoD expression along the entire dorso-medial boundary of the somite. The medial lip cells (stipled shading) are the first to activate MDF expression in the embryo but have repressed *Pax-3*-expression to undetectable levels. The function of these cells is not clear because, according to current models for somitogenesis, the majority of these cells are probably not direct precursors of the early myotome (Kaehn, et al., 1988).

Two new dermomyotome-derived compartments become evident in stage X somites: (4) lateral dermomyotome cells that migrate to the limb bud where they intermingle with the limb mesenchyme cells already there. These migratory dermomyotome-derived cells are strongly *Pax-3*-positive (black shading) and negative for myoD and myf-5; and (5) myotome cells (striped shading), that are differentiated, post-mitotic muscle fibers extending from the cranial to caudal margins of the dermomyotome. They are positive for myoD, myf-5 and contractile proteins, but negative for *Pax-3*. Current models for myotome formation indicate that myotome cells are directly recruited from the cranial-most boundary of region 2 (Kaehn, et al., 1988; Ordahl and LeDouarin, 1992).

Panel F: Transverse view of a wing level somite from a stage 24 HH embryo showing the hypothesized intermingling of *Pax-3*-positive/MDF-negative migratory precursor cells and *Pax-3*-negative/MDF-positive differentiated myocytes.
Limb muscle regions: Migratory somite cells in the limb bud coalesce into dorsal and ventral limb muscle masses and continue to express *Pax-3* at high levels (black shading). Some migratory cells begin to differentiate, (activating MDF and contractile protein expression), and concommitantly repress *Pax-3* expression (striped shading).

Pax-3 expression in paraxial mesoderm

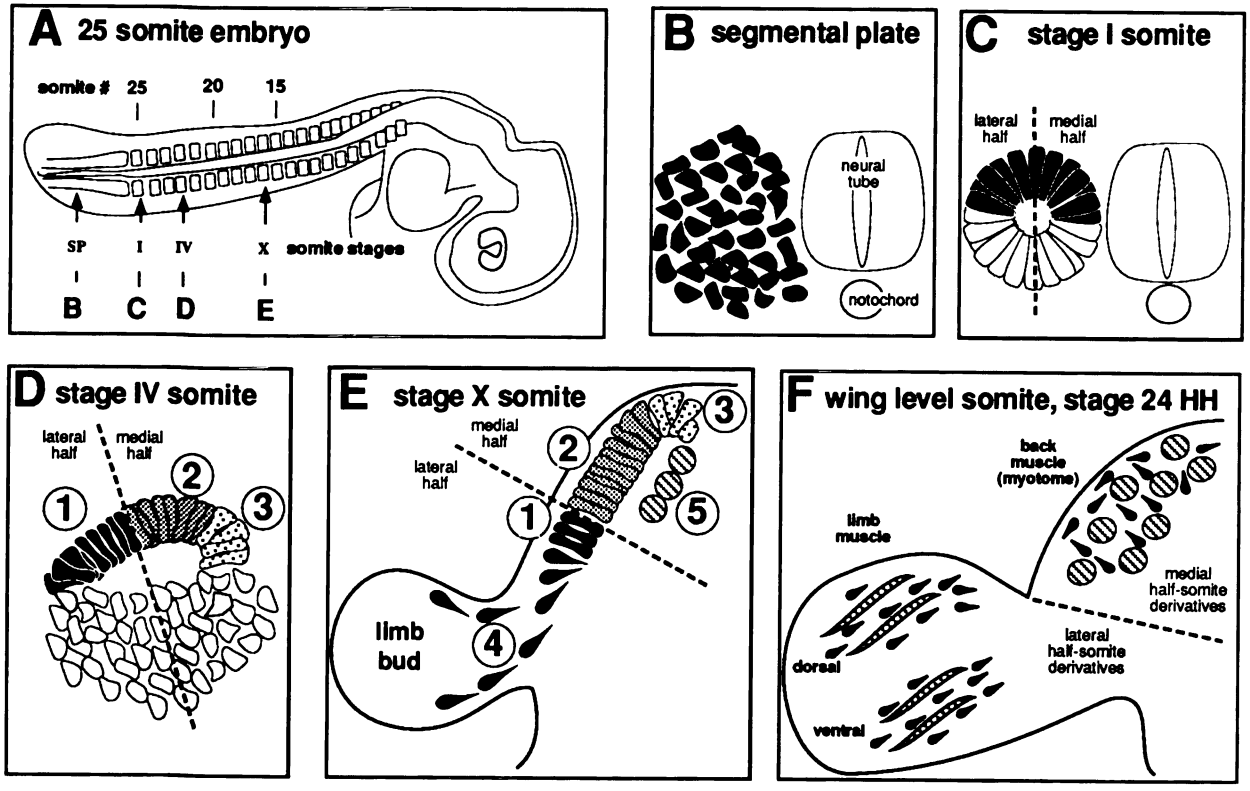

Figure 2.6

CHAPTER THREE

Specification and morphogenesis of early and late myotome precursor cells in the dorso-medial avian somite

Abstract

Previous attempts to characterize the influences necessary for muscle cell specification in the somite have shown that the notochord plays an inhibitory role, and fosters the development of cartilage from cells of the somite (Aoyama and Asamoto, 1988; Brand-Saberi et al., 1993; Goulding, et al., 1993b; Pourquie, et al., 1993; Fan and Tessier-Lavigne, 1994). Whether this effect is produced by conversion of myogenic precursor cells present in the somite to a cartilage fate or simple redistribution of pre-muscle cells away from the influence of the notochord is unknown. We have followed the progeny of grafted quail dorso-medial somite cells that are subject to the influence of grafted notochord by scoring contributions of quail cells to differentiated skeletal muscle and cartilage tissues. Small colonies of disorganized skeletal muscle cells were observed from dorso-medial fragments of the segmental plate and somites II and IV. These colonies were always seen in close association with the spinal nerve; the frequency of muscle colonies increased with stage of somite maturity. Fragments grafted from somite stage X produce large numbers of muscle cells that are organized into recognizable muscle tissue. Large numbers of cartilage cells are produced from all somite stages tested. Thus, commitment to the skeletal muscle phenotype is maintained in a minority of cells in the early somite and segmental plate only in the presence of nervous tissue. Additional events in somite maturation are required to allow proliferation of skeletal muscle precursor cells and formation of organized muscle tissue in the presence of excess notochord signals.

Introduction

Conditional cell specification, the establishment of specific cell types in vertebrate embryos, is believed to result when groups of cells receive extrinsic signals from surrounding tissues, a process referred to as "induction" (Gurdon, 1987). This hypothesis is supported by the observation of "embryonic regulation", in which relatively normal animals develop even after surgical or chemical ablation of specific embryo parts. Additional evidence for induction comes from a variety of experiments which manipulate the juxtaposition of putative signalling and responding tissues (Spemann and Mangold, 1924; Grobstein, 1955; Grobstein and Holtzer, 1955; Jacobson, 1966; Cunha, 1976a; Cunha, 1976b; Saunders et al., 1976). It is unknown whether the process of induction instructs naive cells to adopt new cell fates, or whether small numbers of pre-specified "founder" cells are selectively expanded under an inductive influence (Holtzer, 1978).

Cell specification culminates in specific changes in gene regulatory processes by which cells execute developmental programs in response to inductive signals. In the case of skeletal muscle, recent progress has been made in identifying such cell-intrinsic components of the specification process. The myoD family (MDF) of muscle-specific transcriptional regulatory genes governs contractile protein gene expression (Davis, et al., 1987; Braun, et al., 1989b; Wright, et al., 1989; Miner and Wold, 1990). MDF gene products form heterodimers with members of the ubiquitously expressed E-protein family that bind to regulatory elements of contractile protein gene promoters and activate transcription (Lassar, et al., 1991). Less well understood is the role that MDF family members play in the process of myogenic cell specification. When expression of an MDF is forced by transfection in primary cultures of non-muscle cells, or in cell lines, a heritable ability to differentiate as skeletal muscle is conferred on the daughters of the original

transfectants (Davis, et al., 1987; Weintraub, et al., 1989; Choi, et al., 1990). The maintenance of fidelity to the myogenic differentiation program over several generations in such transfected cells indicates that the transfected MDF provided or elicited a "cellular memory" for the myogenic cell type. Auto-regulatory (and cross-regulatory) loops have been proposed as the molecular mechanism for myoblastic cellular memory (Thayer, et al., 1989) although experiments that disrupt such loops indicate that cellular memory probably requires additional molecular events (Tapscott et al., 1989).

In the vertebrate embryo, myogenic precursor cells are derived from somites (Christ, et al., 1974), compact epithelial spheres that also contain precursor cells for cartilage, dermis and endothelium (Remak, 1855; Rabl, 1888; Christ, 1969; Swalla and Solursh, 1984; von Kirschhofer, et al., 1994). The onset of overt expression of MDF family members is seen in the dorso-medial regions of the stage II somite (Pownall and Emerson, 1992; Ordahl, 1993), consistent with a role for the MDFs in specification of skeletal myogenic precursor cells in the somites. The absence of differentiated muscle in transgenic mice with targetted disruption of MDF genes (Hasty, et al., 1993; Nabeshima, et al., 1993; Rudnicki, et al., 1993) is also consistent with a role for MDFs in the formation of skeletal muscle in the embryo.

Skeletal muscle precursor cells located in different regions of the somite produce muscle for different regions of the body. Precursor cells residing in the dorso-medial regions of the paraxial mesoderm form myotomal muscle adjacent to the body axis, while precursors in the dorso-lateral region either migrate from their axial position to form limb muscle or expand ventrally to form muscle of the body wall (Christ et al., 1983; Ordahl and Le Douarin, 1992). Although spatial patterns for MDF expression are consistent with a future role in muscle tissue formation, little is known about where or when cells expressing MDFs become "determined," or acquire cellular memory that restricts their differentiation to the myogenic path.

Axial structures (neural tube and notochord) of the vertebrate embryo can induce somite cells to undergo cartilage differentiation both in vivo and in vitro (Holtzer and Detwiler, 1953; Waterson et al., 1954; Grobstein and Holtzer, 1955; Lash, et al., 1957; Lash, 1967; Pourquie, et al., 1993). Early studies of cell fate specification in somite compartments focused on the signalling abilities of the notochord. The ability of the notochord to promote cartilage differentiation in vitro was systematically tested on surgically separated somite dermomyotome and sclerotome from stage 18 HH embryos (Cheney and Lash, 1981). The expression of several cartilage markers from sclerotome compartments was enhanced by coculture with notochord, but the dermomyotome responded weakly or not at all to the influence of notochord. The cartilage promoting effects of notochord have also been tested in vivo. A supernumerary notochord grafted between the neural tube and the segmental plate of the chick embryo suppresses the formation of myotomal muscle, enhances the formation of axial cartilage in the dorsal axial domain, and induces the expression of Pax1 mRNA (a marker for sclerotome) in close proximity to the ectopic notochord (Brand-Saberi, et al., 1993; Pourquie, et al., 1993; Fan and Tessier-Lavigne, 1994). The cells of the segmental plate appear to be uniquely susceptible to this ventralizing influence of the notochord, because mature somites produce myotomal muscle when juxtaposed with ectopic notochord (Pourquie, et al., 1993). Thus, cells within the segmental plate that are fated to become muscle have not yet acquired cellular memory, a key feature of cell determination. Moreover, somite rotation and inter-specific grafting experiments (Aoyama and Asamoto, 1988; Aoyama, 1993) indicate that prospective muscle-forming cells in the early somite can switch fates to form cartilage but lose this ability at later stages of somite development.

Although cell determination is a concept that dates to the earliest days of experimental embryology, unequivocal assays for determined cells have been elusive. Classically defined, determined cells in the embryo are those that "continue to develop

autonomously after grafting to any other region of the embryo"; and this state is believed to be "clonally inherited, since it must be independent of environment" (Slack, 1983). Grafting experiments designed to identify determined cells must take into account both the type of signalling influence to which precursor cells are exposed, and temporal changes in the response of precursor cells to the signal. For example, previous experiments in which presumptive muscle precursor cells were grafted to the limb bud indicate that "determined" muscle precursor cells already exist within the segmental plate (Wachtler et al., 1982; Krenn, et al., 1988), a result in apparent conflict with the notochord implantation results described above. Therefore, although segmental plate cells may continue to develop into muscle in a neutral or a permissive environment, the notochord is able to induce such cells to form cartilage. In other words, such precursor cells in the segmental plate have acquired "competence" to form muscle in a neutral environment but they may not yet have acquired "memory" that makes them refractory to induction into other cell types.

We have developed a novel, alternative assay for myogenic determination that exploits the signalling environment of the somite to assess the acquisition of myogenic cell memory. Paraxial mesoderm cells are challenged in vivo with a powerful chondrogenic inducer, the notochord. In this assay, fragments of quail paraxial mesoderm are transplanted into chick host embryos under conditions in which they are surrounded by notochord signals. We reasoned that myogenic precursor cells that are determined should be unresponsive to these signals and should continue to make muscle while undetermined cells should respond to the notochord challenge by forming cartilage. By taking paraxial mesoderm fragments from the dorso-medial quadrant we restrict the challenge to only myotomal precursor cells. In addition, by taking dorso-medial quadrant cells from different stages of paraxial mesoderm development we are able to correlate the acquisition of determination with cellular changes in somite development as well as the expression of key myogenic determination markers, such as the myogenic bHLH factors. Our results

demonstrate that myogenic determination is acquired progressively by cells of the dorso-medial quadrant during paraxial mesoderm development. Moreover, there are qualitative and quantitative changes in the determined cells that may reveal important clues to the cellular and molecular processes of muscle formation in the embryo.

Methods

Embryo surgery

Fertile quail eggs were obtained from Strickland Quail Farm (Pooler, GA). Fertile chick eggs were obtained from Western Scientific Products (Sacramento, CA). Egg incubation and general embryo surgical procedures were performed as described (Ordahl and Christ, 1996; Williams and Ordahl, 1996). Notochords from stage 15HH chick embryos were prepared as previously described (Pourquie, et al., 1993) and kept in holding solution (2% fetal calf serum in Tyrode's solution) until transplantation. All donor tissue fragments were implanted in the right-hand side of the host embryo at the level of the developing limbs (somites 15-20).

Preparation of somite dorso-medial quadrants from quail embryos.

Two day quail embryos (stage 12-13HH) were pinned ventral side up in dissection dishes and a longitudinal midline incision was made in the endoderm adjacent to somite stages I-XV (Figure 3.1A). Adherent tissues, such as aorta or endoderm were teased away using a microscalpel to expose the notochord and ventral surface of the somites. A longitudinal incision was then made through the somites to separate their medial and lateral halves. Sclerotome tissue of the ventromedial quadrant of the somite was then removed by aspiration with a micropipet after a brief treatment with collagenase (0.5%, Sigma cat no. C0130). The remaining somite dorso-medial quadrant (Figure 3.1B) was then teased away

from the underlying ectoderm using the flat side of a microscalpel and transferred to holding solution using a P-20 pipetman until ready for implantation into the host.

In order to evaluate the quality of the surgery, some donor embryos were fixed after the excavation of ventral sclerotome, while the dorso-medial somite quadrant was still in place in the donor embryo. The embryo was then sectioned and stained with the MF20 antibody (Developmental Studies Hybridoma Bank, University of Iowa), which recognizes skeletal muscle myosin, to assay the quality of sclerotome removal and the presence of already differentiated muscle cells in the dorso-medial somite fragment.

Cell Numbers in Somite Fragments

A series of donor fragments from somites II (n=9) and XI (n=8) were prepared as above, and used to estimate the number of quail cells initially grafted to the host embryo. Cells were incubated in a 20 ul droplet of 25 ug/ml Hoechst stain 33342 (Molecular Probes, Eugene, OR) in Tyrode's solution at 37C for 5 minutes, on a microscope slide. An additional 20 ul of 0.5% collagenase and 20 ul pancreatin (1X) was added and the fragment reincubated for an additional 5 minutes. A coverslip was used to squash the fragment so that the nuclei were separate and distinct, and an image of the separated nuclei was collected on the Zeiss Axiophot under epi-illumination. The image was printed and the number of nuclei counted manually.

Preparation of Host Embryos

Chick embryos containing between 15 and 20 somites (stage 12-13HH) were used as hosts in this study (Figure 3.1C) and were prepared for surgery in ovo as described (Ordahl and Christ). The target site for surgery in each case was the stage I somite which was exposed via an incision in the ectoderm between the neural tube and the paraxial

mesoderm comprising somites I-V and the segmental plate (Figure 3.1D). A longitudinal incision was then made through somite I and its entire medial half removed (Figure 3.1E).

Implantation of donor somite fragments and notochord fragments

The quail donor somite fragment was transferred to the chick donor by micropipet (Figure 3.1E) and placed deeply into the medial position of somite I using a microscalpel (Figure 3.1F). Once the donor somite fragment was securely in place, two donor notochords were implanted in the groove between the neural tube and somites (Figure 3.1G). Preliminary experiments were identical except that only one donor notochord was implanted.

Care was taken in several aspects of this procedure. First, donor notochords were implanted over the donor somite fragment so that they spanned a 6 somite-equivalent distance cranially and caudally relative to the implanted donor somite fragment. This was done to ensure that signals from the notochord surround, as completely as possible, the donor somite fragment being tested. Second, donor somite fragments were transplanted into host embryos within 40 minutes of removal from donor quail embryos. Third, donor notochords were implanted within 90 minutes of being removed from donor chick embryos. Fourth, two donor notochords were implanted to restrain the grafted quail cells to the ventral-epaxial domain, to prevent them from coming under the muscle promoting influence of tissues of the dorsal neural tube and skin ectoderm, while at the same time extending the range of effective notochord signalling.

Analysis of cell division by undifferentiated muscle precursor cells

In some cases, donor fragments from stage IV or stage XII somites were incubated for 90 minutes at 37C in 100uM BrdU (Sigma cat no. B-5002) in Tyrode's solution. After two rinses, the labelled fragment was implanted as above and the chimeric embryo was

incubated for an additional 24 hours. The number of differentiated muscle cells that incorporated BrdU prior to differentiation was estimated by immunohistochemical stain on adjacent sections with the MF20 antibody (DSHB, Iowa City, IA) and an anti-BrdU antibody (IU-4, Caltag Laboratories) that is specific for BrdU incorporated into DNA.

Histology

Harvested embryos were fixed with Carnoy's fixative, embedded in paraffin, and sections cut at 7μm. Adjacent sections were stained with the Feulgen reaction (Le Douarin) or the QCPN anti-quail antibody (Developmental Studies Hybridoma Bank) to identify quail cell nuclei; the MF-20 anti-myosin antibody (Developmental Studies Hybridoma Bank) to identify differentiated skeletal muscle; or hybridized to ^{35}S labelled cRNA probes for Pax1, Pax3, myoD and myf5; all as described (Williams and Ordahl, 1994). The contribution of graft-derived quail cells to mesenchyme, muscle and cartilage was determined by visual inspection using a Zeiss Axiophot microscope. Numbers of quail nucleoli present in muscle tissue were estimated by counting nucleoli on Feulgen stained sections that were present in MF20 staining tissues on the adjacent section. Numbers in cartilage tissue were estimated on Feulgen stained sections by simply counting nucleoli present within the cartilage model. Images were collected using the DEI 470 Optronics CCD video camera system (Goleta, CA) and processed using Adobe Photoshop 3.0 software.

Analysis of chimeric embryos

Embryos were histologically evaluated at 5 hours, 24 hours and 6 days post-surgery to determine the position of the grafted somite fragment in relation to host structures and donor notochords. Figure 3.2A shows the arrangement of these tissues 5 hours after implantation of the dorso-medial quadrant of a stage IV somite followed by

implantation of two donor notochords. Quail cells are present in mesenchyme and in an epithelioid structure closely apposed to the ventral neural tube. The 24 hour specimen (Figure 3.2B) shows a greatly expanded mesenchyme with some cells clustered near the notochord. No recognizable host dermomyotome epithelium is evident on the operated side by 24 hours post-surgery. A small cluster of contiguous graft cells is seen.

Six days after surgery, adjacent sections from 46 chimeras were analyzed for the presence of quail cells in muscle and cartilage. The results of these experiments are summarized in Table 3.1. Figures 3.2C & D show an immunohistological analysis of cross sections through the grafted region of a chimera. The presence of quail cells is detected with the QCPN anti-quail antibody (panel c) and striated muscle myosin is detected with the MF20 antibody (panel d). Ectopic cartilage is present on the operated side of these chimeras, in the form of distorted vertebral bodies and lamina as seen previously (Brand-Saberi, et al., 1993; Pourquie, et al., 1993). Extensive incorporation of quail cells into these cartilage structures (arrows) surrounding the neural tube was seen in all but one of the 46 chimeras evaluated (Table I). Host-derived and donor-derived cartilage cells in chimeric structures were segregated into well-defined domains.

Donor muscle tissue was found in either of two distinct domains in chimeric embryos. In 25% of single and 15% of double supernumerary notochord chimeras donor muscle tissue was located dorsal to the implanted notochord(s) (Figure 3.2D). Such dorsally-located donor muscle tissue appeared in some cases to lie partially inside the vertebral lamina surrounding the neural tube.

We reasoned that dorsally-displaced somite cells might have received muscle-promoting signals arising from the dorsal neural tube and/or skin ectoderm, (Christ, et al., 1992; Buffinger and Stockdale, 1994; Fan and Tessier-Lavigne, 1994; Buffinger and Stockdale, 1995; Munsterberg, et al., 1995; Munsterberg and Lassar, 1995; Stern, et al., 1995; Stern and Hauschka, 1995) thereby confounding the cartilage inducing influence of

the implanted notochords. Moreover, the presence or absence of dorsal donor muscle in these 7 chimeras was unrelated to somite stage from which the dorso-medial quadrant was isolated (see Table I). For these reasons, we did not analyze muscle development in these specimens further.

In the remaining 39 chimeras, donor quail cells were restricted to the ventral region of the operated side (Figure 3.2D), a region of maximal notochord influence. The appearance of donor muscle tissue and myocytes in this region was related to the developmental age of the grafted dorsomedial quadrant (see Results).

Results

Anatomy of the notochord challenge environment

Initial experiments were designed to test the ability of cells within the dorso-medial quadrant of the somite to switch fates, from presumptive muscle and/or dermis, to cartilage. Single supernumerary notochords were implanted ectopically in 31 chick embryos as previously described (Pourquie, et al., 1993) except that the medial half of the host stage I somite was replaced with the dorso-medial quadrant of quail somites from stages I to X (Ordahl, 1993). Supernumerary notochords were deployed both as a source of cartilage converting signal and as an obstacle to prevent the migration of graft-derived cells into the dorsal epaxial domain, where they might receive muscle promoting influences from the dorsal neural tube or dorsal ectoderm. However, in many cases graft-derived cells were displaced dorsally in the chimeras.

To maximize notochord signalling, and to improve restraint in the ventral epaxial domain, an additional 46 chimeras were prepared in which the donor test fragment was implanted deep within the medial somite compartment close to the host notochord with two implanted donor notochords overlaid. The quail nucleolar marker was used to identify

graft-derived cells in cartilage, muscle, paravertebral mesenchyme, mesenchyme surrounding the neural tube, the connective tissue sheath surrounding the spinal nerve, and the endothelial lining of several blood vessels (data not shown).

Chondrogenic potential within the somite dorsomedial quadrant

Quail-derived cartilage cells were detected in 76 out of 77 specimens (see, for example, Figure 3.2d), and from all stages of donor somite fragments tested. In all but 2 of these cases, cartilage cells were present in large numbers. While we cannot rule out the possibility that sclerotome precursor cells have also been carried along with the dorso-medial fragments of mature somites (stages XI-XIII, see figure 8 and below), it is unlikely that sclerotome was included with the dorso-medial quadrants of early somites (stages II & IV). These results are consistent with the notion that cells with chondrogenic potential persist within the somite dorso-medial quadrant, that the cartilage fate is brought forth by exposure to the powerful inductive influence of the notochord, and are in agreement with previous findings (Pourquie, Aoyama).

Muscle formation is different in the dorsal and ventral epaxial domains

In 39 chimeras the donor-derived cells remained ventral to the implanted notochords, the position in which notochord signalling is expected to be maximal (see Figure 3.2 and methods). The appearance of muscle in this ventral epaxial domain was correlated with the stage of somite development from which the donor dorsomedial quadrant fragment was derived. The data from these experiments is summarized in Table 3.1 and accompanying histological analysis described in detail below.

In seven cases included in this study, somite cells escaped confinement in the ventral epaxial domain bounded by three notochords and were displaced dorsally in close proximity to the dorsal neural tube (Table 3.1). Figure 3.2 demonstrates the response seen

in this minority of cases. Panel C shows the graft-derived cells stained with the QCPN antibody; in panel D, skeletal muscle cells are stained with the MF-20 antibody. Muscle tissue composed of thousands of quail cells formed large, elongated myofibrils that appear to run transverse to the body axis. They are roughly equal in number to the quail cells found in the cartilage model of the neural arch. Dorso-medial quadrant somite cells from each of the different maturational stages tested were capable of this behavior.

Small myoclusters from the dorso-medial quadrant of the segmental plate.

Figure 3.3 shows two adjacent cross sections of a chimeric embryo made by grafting the dorso-medial quadrant of the segmental plate in place of the medial half of somite I and challenging with two supernumerary notoochords. Panel A is a Feulgen stained section identifying graft derived quail cells. A general morphological distortion on the operated side is seen, with a large ectopic cartilage bulge in the vertebral cartilage model. Neural tube and notochords are marked as in the legend. Panel B shows the location of differentiated skeletal muscle cells by immunohistochemical identification with the MF20 antibody. The majority of segmental plate dorso-medial quadrant chimeras (75%) gave rise to abundant mesenchyme (Figure 3.3 C & D) and cartilage (E & F), but no muscle (see Table 3.1). These results confirm the earlier findings of Pourquie et al., who postulated a conversion of dorso-medial paraxial mesoderm to cartilage when notochord was grafted adjacent to the segmental plate.

Two chimeras (25%) formed small disorganized clusters of differentiated myocytes. We refer to these small clusters of differentiated myocytes as "myo*clusters*" rather than "*colonies*" to avoid misunderstandings about the (unknown) number of founder cells present in the donor segmental plate fragment. A histological analysis of small myoclusters is presented below.

Myoclusters are characteristic of dorsomedial quadrants from stage II and stage IV somites

Small myoclusters were detected in half of the chimeras resulting from transplantation of stage II somite dorso-medial quadrants (Table 3.1). In the remaining seven chimeras, no muscle was formed. Figure 3.4 shows the analysis of a chimera containing a single small myocluster. Morphologically-identifiable donor muscle tissue was not grossly evident, but the very small, disorganized cluster of differentiated muscle cells could be detected by anti-myosin staining (panel B). Colocalization of the quail nucleolar marker (panel C) and myosin staining (panel D) demonstrate that many, and possibly all, of the myonuclei in this cluster are quail-derived. Three features of these myoclusters are of interest:

First, small myoclusters contain fewer than 20 individual differentiated quail myocytes, each with a single nucleus.

Second, myoclusters are unorganized. Individual myocytes are neither bipolar nor aligned with neighboring myocytes. Cross striations are not evident. Quail myocytes are interspersed with, and morphologically indistinguishable from surrounding mesenchyme cells (see also below).

Third, myoclusters are always found in proximity to peripheral nervous tissue. This is evident in Figure 3.4A, B where the donor myocluster is located near a nascent vertebral foramen through which the spinal nerve passes. All 15 cases of small myoclusters were found in comparable proximity to peripheral nerve tissue.

Approximately half of the chimeras resulting from transplantation of stage IV somite dorso-medial quadrants gave rise to small myoclusters with the same characteristics as those described above for stage II somite dorso-medial quadrants. Three stage IV somite dorso-medial quadrant chimeras, however, gave rise to larger "medium-sized" myoclusters estimated to contain between 100 and 150 donor myonuclei. Otherwise, these

myoclusters had the same characteristic disorganization and proximity to spinal nerve as noted above for small myoclusters.

Muscle tissue from the dorso-medial quadrants of somites stages XI-XIII

Dorso-medial quadrants from the majority (75%) of stage XI-XIII somites formed large, anatomically distinguishable muscle tissue in a position ventral to the implanted notochords (Figure 3.5B). Such muscle tissue consisted predominantly of quail nuclei arranged in bipolar, multinucleate myotubes (panel C & D). In peripheral regions, chick cells also were seen to contribute to muscle tissue in some cases. Cross striations (not shown) and centrally located nuclei are clearly evident. These muscle masses showed well-organized fiber bundles (panel B) containing thousands of nuclei, organized separately from other surrounding tissues. In the example shown, as in most other cases, the direction of donor fiber alignment was perpendicular to the host body axis. There was no correlation between the formation of these ectopic muscle tissue masses and the proximity of a spinal nerve (see below).

Characterization of mesenchyme cells surrounding myoclusters

As indicated above, donor myoclusters reside within mesenchyme tissue that contains a mixture of both host and donor cells. We wanted to know if donor mesenchyme cells might be arrested at some earlier stage of muscle specification. Therefore, in situ hybridization was used to determine if surrounding, donor-derived mesenchyme cells express early muscle specification markers. Figure 3.6 shows adjacent cross sections of a HH 30 chimeric embryo containing a graft of a quail stage IV somite dorso-medial quadrant. Panel B shows an enlargement of the area marked in panel A where mesenchyme is populated with donor cells. This region is negative for myosin protein (panel C), and for the messenger RNAs for myoD (D), myf 5 (E), and pax-3 (F). We

conclude that the surrounding mesenchyme does not represent muscle precursor cells arrested at an early stage of myogenic specification definable by these markers.

Early response to the challenge

It is unknown whether the unusual microenvironment of the notochord challenge is conducive to the survival of muscle precursor cells. To verify that muscle precursor cells in the dorso-medial somite can differentiate in close proximity to notochord, we analyzed chimeras for the presence of nascent muscle tissue 24 hours after grafting stage XI dorso-medial quadrants into the notochord challenge. Figure 3.7A shows that cells derived from the grafted quail somite fragment remain ventral to the implanted notochords. Those cells derived from the graft that are clustered in a group express sarcomeric myosin protein (panel B); myf 5 and myoD mRNAs (Panels C & D, respectively) but neither Pax-3 nor Pax-1 mRNAs (Panels E & F, respectively). Analysis of chimeras produced with stage IV somite fragments showed a similar rapid appearance of differentiated muscle, but with many more graft-derived cells present in loose mesenchyme (data not shown). These results demonstrate that robust muscle differentiation can occur in close proximity to donor and/or host notochords.

Cellular complexity of grafted tissue

To estimate the number of quail donor somite cells that were initially placed in the challenge, dorso-medial fragments of somites II (n=9) and XII (n=8) were labelled with Hoechst stain and nuclei counted after image collection from the fluorescent microscope (data not shown). Dorso-medial fragments from somite II contained approximately 280 nuclei (± 37 SEM), whereas dorso-medial fragments from somite XII contained 700 nuclei (± 103 SEM).

The cellular complexity of dorso-medial somite grafts that were placed into the challenge was evaluated by immunohistochemistry on cross sections of quail donor embryos prepared in the same way as for graft preparation. Figure 3.8 shows adjacent cross sections through donor somites immediately after excavation of the ventro-medial somite or underlying sclerotome cells. In panels A and B, cross-sections through somites II and IV are shown. The ventro-medial somite cells and the somitocoel cells from the medial half of the somite have been removed, leaving the overlying dorsal epithelium intact. The dermomyotome is normally cut at the location of the arrow, separating the medial from the lateral half. Panel C is a cross-section through a stage XI somite, with most of the underlying medial sclerotome cells removed. A few mesenchymal cells remain between the dorso-medial lip of the dermomyotome and the neural tube. Panel D is a cross-section stained with the MF20 antibody, showing that already differentiated skeletal muscle cells (arrowhead) are contained within the dorso-medial fragment of the somite that will be transferred to the host embryo.

Mitotic skeletal muscle precursors in the dorso-medial somite

A mitotic labelling experiment was used to determine whether the differentiated muscle cells in grafts from older somites were derived from mitotically active precursors or from post-mitotic cells. Dorso-medial fragments from stage XII somites were incubated with the thymidine analog BrdU for 90 minutes prior to implantation, and collected 24 hours after placement into the notochord challenge (Figure 3.9). Post-mitotic myocyte precursor cells in the graft would not be expected to incorporate this label, while mitotically active myotome precursor cells would be expected to uptake the label and eventually form differentiated muscle with BrdU labelled nuclei.

MF20 stained skeletal muscle tissue (panels A and B) contains quail cells (panel C) that are labelled with BrdU (panel D). The appearance and number of cells present in BrdU

treated grafts from the stage XI somite was indistinguishable from untreated grafts collected at this age. Therefore, while some already differentiated skeletal muscle cells are present in the initial donor fragment (as shown in Figure 3.8D) undifferentiated muscle precursor cells that have not yet withdrawn from the cell cycle progress through at least one S-phase prior to differentiation after 24 hours in the notochord challenge environment.

Discussion

An in vivo assay to score cell fate decisions between cartilage and skeletal muscle

We have developed a novel determination assay which challenges prospective skeletal muscle precursor cells with a powerful chondrogenic inducer, the notochord. Previous work has established the notochord as the signal that induces ventral somite cells to form sclerotome, the precursors of vertebral cartilage (Holtzer and Detwiler, 1953; Waterson, et al., 1954; Grobstein and Holtzer, 1955; Lash, et al., 1957; Lash, 1967; Brand-Saberi, et al., 1993; Pourquie, et al., 1993; Fan and Tessier-Lavigne, 1994). The assay is performed in vivo, with marked prospective skeletal muscle precursor cells surrounded by grafted notochords. The in vivo environment is preferred to the in vitro environment for several reasons. The growth of developing muscle tissue can be assessed in tissue sections more accurately than in culture. Full differentiation of muscle cells into organized tissue is recognizable in sections, but is not seen in the culture environment. Unexpected interactions with surrounding tissues can be described that are not possible in defined in vitro systems.

This assay is an improvement on the original definition of determination assays in that it rigorously tests temporal changes in the response of muscle precursor cells to signals that are known to influence them in early stages of development in the embryo. We reasoned that undetermined somite cells would differentiate into chondrocytes in this assay

while determined myogenic precursor cells that have acquired phenotype memory would no longer respond to the cartilage-inducing signals of the notochord and continue to differentiate as muscle. By assaying prospective muscle precursor cells from different stage somites, the temporal acquisition of muscle lineage memory can be assessed.

Persistent chondrogenic potential in the dermomyotome.

Dorsal half-somite transplant experiments indicate that cells in the dorsal half of the somite are fated to form skeletal muscle but not cartilage (Christ, et al., 1992; Aoyama, 1993). Medial half-somite transplant experiments indicate that the medial portion of the dorsal half will give rise to the muscle and dermis of the epaxial domain (Ordahl and Le Douarin, 1992). Despite these fate assignments under normal conditions in vivo, we found that the dorso-medial quadrant fragments of the paraxial mesoderm were able to form large amounts of cartilage in response to the notochord challenge. This ability was unrelated to the age of the paraxial mesoderm tested and large amounts of cartilage were obtained from the dorso-medial quadrants of mature somites which formed well organized muscle tissue. We conclude, therefore, that chondrogenic potential remains within the cells of the medial dermomyotome possibly even at advanced stages of dermomyotome development.

Such chondrogenic potential of the dermomyotome was less evident when intact somites were analyzed previously. For example, somite-rotation experiments indicate that cells of the dermomyotome could be re-specified to form sclerotome at early somite stages (I & II) but were resistant to such influences by somite stage III (Aoyama and Asamoto, 1988). In addition, dorsally grafted notochord induces ectopic cartilage only from segmental plate but not from formed somites (Pourquie, et al., 1993). The finding here that chondrogenic potential can be elicited from dorso-medial quadrant fragments at least as old as stage IV (and possibly as old as stage XIII) indicates that the surgical disruption of the somite epithelium may reduce the resistance of somite cells to cartilage inducing signals

from the notochord that alter the fate of the dermomyotome. Thus, an intact somite organization may maintain commitment of unspecified dermal and/or skeletal muscle precursor cells within the dorso-medial somite. Disruption of the epithelium and/or overall somite organization may make dorso-medial somite cells more susceptible to cartilage conversion by notochord signals.

Quantitative aspects of myogenic precursor cells in the dorso-medial somite.

Differentiated muscle cells were detected in chimeras formed from the dorso-medial quadrants of somites of all ages tested but the appearance of such cells was quantitatively and qualitatively related to the developmental age of the donor somite (Figure 3.10, and Table 3.1). 25% of the segmental plate fragments tested gave rise to differentiated myocyte clusters containing less than 20 cells. A progressive increase in the number of myocytes that differentiated in the presence of notochord was also seen. Dorso-medial quadrants from segmental plate and stage II somites yielded clusters of less than 20 individual myocytes, while a minority of those from stage IV somites gave rise to larger clusters containing up to 150 myocytes. Large numbers of muscle cell nuclei (>1000) resulted from transplantation of dorso-medial quadrants of older somites (stages XI-XIII). Incorporation of BrdU into the nuclei of muscle from older somite grafts indicates the presence of mitotically active muscle precursors at the time of grafting. These combined results indicate that during somite maturation, increasing numbers (or increased mitogenic capacity, or both) of determined myogenic precursor cells arise in the dorso-medial somite (see also below).

Myogenic cells can be cultured from dispersions of avian segmental plate and early epiblast (Pourquie, et al., 1993; George-Weinstein et al., 1994; George-Weinstein, et al., 1996); in the case of the segmental plate, the fraction of cells with myogenic capacity is very high. Segmental plate cells only infrequently produce a small number of myocytes in

the notochord challenge, suggesting that repressive mechanisms within the segmental plate are evident in the in vivo assay used here that do not appear to operate in vitro. Myogenic repressors such as twist (Hebrok et al., 1994; Fuchtbauer, 1995) and notch (Reaume, et al., 1992; Kopan, et al., 1994) are expressed in the rostral tip of the segmental plate immediately prior to somite formation. The effect of neural tissue adjacent to small myoclusters may be to relieve this repression.

Morphogenetic aspects of dorso-medial myotome precursors

In addition to the progressive increase in numbers of differentiated skeletal muscle cells produced from the dorso-medial somite discussed above, the assay also revealed qualitative differences in the type of muscle cell that differentiates from the dorso-medial somite at early and late stages of maturation (Figure 3.10). Dorso-medial quadrants from the segmental plate, stage II and stage IV somites formed only small clusters of differentiated myocytes that were interspersed with non-muscle mesenchyme cells. Myocluster cells were mononucleate and multipolar resembling, in
many ways, differentiated muscle cells seen in culture (Rong, et al., 1992; Buffinger and Stockdale, 1994; Buffinger and Stockdale, 1995; Gamel et al., 1995; Munsterberg, et al., 1995; Munsterberg and Lassar, 1995; Stern, et al., 1995; Stern and Hauschka, 1995). These qualities reflect a low level of tissue organization.

Dorso-medial quadrants from stage XI-XIII somites, on the other hand, gave rise to masses of muscle tissue that were separate from host muscle and which had distinct boundaries separating them from surrounding host tissue. This muscle tissue was highly organized with bipolar myotubes containing multiple, centrally located nuclei. Myotubes were organized in parallel arrays within bundles and adjacent bundles showed parallel organization and orientation. Interestingly, donor myotubes were often oriented

transversely, perpendicular to the orientation of host epaxial muscle fibers which are always parallel to the embryonic axis.

These findings indicate that a major difference between precursor cells of the older somite compared to precursor cells of younger somites is the ability to generate organized, differentiated skeletal muscle tissue. This may reflect the emergence of a separate myogenic lineage with properties greatly different from the muscle precursor cells in early somites, or simply an additional capacity acquired by early muscle precursor cells during maturation within the somite. It is also possible that a separate population of non-muscle connective tissue cells that enable muscle morphogenesis arises in the later somite. Connective tissue cells with this ability are known to organize the form of skeletal muscles in the limb.

Nerve dependent myoclusters

A noticeable attribute of myoclusters was their obligatory association with nerve tissue. Although graft-derived quail cells were widely dispersed, myoclusters were only found in close apposition to the spinal nerve indicating that myocluster induction and/or survival is probably nerve-dependent. The spinal nerve may weakly mimic the muscle promoting ability of the neural tube (Vivarelli and Cossu, 1986; Kenny-Mobbs and Thorogood, 1987; Buffinger and Stockdale, 1994; Buffinger and Stockdale, 1995; Munsterberg, et al., 1995; Munsterberg and Lassar, 1995; Stern, et al., 1995; Stern and Hauschka, 1995). By contrast, no correlation was found between the formation of muscle tissue arising from transplanted dorso-medial quadrants of stage XI-XIII somites and proximity to the spinal nerve. Nerve-dependent and nerve-independent myogenic differentiation is also a property of different myoblast lineages present during early and late limb bud development (Bonner, 1978).

The apparent neural dependence of myoclusters from segmental plate and somites II and IV, raises questions about the functional significance of low level expression of MDFs in the segmental plate (Kopan, et al., 1994; Lin-Jones and Hauschka, 1996) and the onset of overt MDF expression in somites II and IV (Pownall and Emerson, 1992). If the few persistent skeletal muscle cells seen in 8 day old chimeras are indeed the cells that initially expressed MRFs in the paraxial mesoderm (Pownall and Emerson, 1992; Kopan, et al., 1994; Lin-Jones and Hauschka, 1996), it appears that additional neural influence is required to maintain their commitment to the skeletal muscle fate when challenged with the powerful cartilage inducing influence of the notochord.

Correlation to the expression of transcription factors

An early marker of the skeletal muscle lineage, Pax3, is expressed throughout the segmental plate of the early embryo, and becomes restricted to the precursor compartment of the myotome, the dermomyotome, during maturation of the somites (Bober et al., 1994b; Goulding, et al., 1994; Williams and Ordahl, 1994). Cells from the dorso-medial segmental plate only infrequently form differentiated skeletal muscle cells under the influence of notochord signalling (Pourquie, et al., 1993), and this study) and instead are converted to the cartilage fate. Thus it appears that expression of Pax3 in cells of the paraxial mesoderm may function upstream in the specification progression, but does not irreversibly commit cells resident in the paraxial mesoderm to the muscle fate.

Myogenic bHLH transcripts are detectable by in situ hybridization as early as somite stage II (Pownall and Emerson, 1992). Thus, overt expression of this transcription factor mRNA correlates well with the appearance of functionally determined cells detected by this assay. In 50% of the cases, the dorso-medial quadrant of stage II somites gave rise to small myoclusters (20 cells or less). Myf-5 mRNA expression is first detectable by in situ hybridization in the dorso-medial quadrant of stage IV somites (Pownall and Emerson,

1992). Stage IV dorso-medial quadrant grafts yielded both a higher frequency of skeletal muscle cell differentiation, and in a few cases larger numbers of differentiated myocytes (up to 150). Since such precursors are capable of limited mitotic expansion in the challenge assay (see Figure 3.10), the onset of overt myf5 transcription correlates well with the enhanced mitotic potential in myogenic precursor cells in the dorso-medial somite. Myogenic bHLH transcripts can be amplified from virtually any tissue of the early embryo, suggesting that low-level transcription of these factors is inconsequential in the embryonic context (Kopan, et al., 1994; Lin-Jones and Hauschka, 1996).

The effect of notochord signalling on muscle cell specification

Graft derived quail cells that remain clustered in a group can differentiate as skeletal muscle even when immediately adjacent to supernumerary notochords (Figure 3.7), suggesting that they are highly resistant to destabilizing influences of the notochord that might draw them out of the myogenic lineage. Expression of myoD, myf5 and myosin heavy chain is clearly evident in these cells, while the expression of Pax3 mRNA is repressed below background levels, as seen in the undisturbed somite (Williams and Ordahl, 1994) at the onset of expression of myoD. This repression of Pax3 mRNA expression has been reported previously in response to notochord signals, but has not been attributed to the differentiation of skeletal muscle. There is some recent evidence suggesting a role for the notochord, or notochord derived factors, in the induction of the muscle cell type in the paraxial mesoderm (Buffinger and Stockdale, 1994; Johnson et al., 1994; Buffinger and Stockdale, 1995; Hammerschmidt, et al., 1996; Pownall et al., 1996; Weinberg, et al., 1996). Experiments in the zebrafish have demonstrated a requirement for an intact notochord for proper myoD expression (Weinberg, et al., 1996). Proper signalling by the notochord derived factor *sonic hedgehog* is required for myoD expression (Hammerschmidt, et al., 1996), and over-expression studies in chick embryos have shown

upregulation of myoD in response to *sonic hedgehog* (Johnson, et al., 1994). However, targeted disruption of the *sonic hedgehog* gene in the mouse embryo does not affect the onset of expression of myoD and myf5, but does result in a deficiency of skeletal muscle (Chiang, et al., 1996). Thus, the notochord may be a source of trophic factors that enable the expansion of small groups of already specified muscle precursor cells (Gordon and Lash, 1974; Bober et al., 1994a). The weak muscle differentiation response of cells in coculture with notochord (Gordon and Lash, 1974; Bober, et al., 1994a; Buffinger and Stockdale, 1994; Buffinger and Stockdale, 1995) and the initiation of muscle differentiation as judged by myoD expression in surgically manipulated embryos (Pownall, et al., 1996) might be explained by this type of mechanism. It has been demonstrated that *sonic hedgehog* stimulates mitogenesis in somite explant cultures (Fan et al., 1995).

Unclustered, mesenchymal quail cells in both early and late somite grafts extinguish the expression of Pax3 mRNA under notochord influence within 24 hours and begin to express the sclerotomal marker Pax1. When exposed to excess notochord signals, these cells may be induced to separate from the somite epithelium and become susceptible to cartilage inducing signals from the notochord. The number of graft derived mesenchymal cells from early somite grafts appears to be far greater than the number of mesenchymal cells from grafts of late somites (data not shown). Although this difference could reflect the limitations of surgical separation of dermomyotome from sclerotome, it is possible that the epithelium of the late somite is more resistant to the mesenchymalizing influence of the notochord. Thus, a continuous somite epithelium that expresses Pax3 may be required to generate organized myotomal muscle in the somite. Under the influence of excess notochord signals, this epithelium is consumed rapidly (within 24 hours in this assay) by the production of differentiated skeletal muscle tissue.

Cellular dynamics of myogenic commitment in the dorso-medial somite

Both young and old somite grafts contain cells that differentiate as skeletal muscle rapidly (within 24 hours) in the notochord challenge. The increase in numbers of differentiated muscle cells generated by progressively older somites may have resulted from:

(1) the ability of a relatively constant number of myogenic precursor cells to survive in the notochord challenge environment that is acquired as they mature within the somite.

The coincidence of the spinal nerve with myoclusters from the segmental plate and the early somites suggests that trophic factors supplied by the nerve might be an important requirement for myocluster survival. It is possible that the paucity of muscle cells found in grafts of early somites is caused by the death of muscle precursor cells in the notochord challenge, due to their isolation from muscle specific trophic factors in other parts of the embryo. Nerve tissue is known to promote the differentiation of muscle from somites (Vivarelli and Cossu, 1986; Kenny-Mobbs and Thorogood, 1987) and a lineage of nerve-dependent myoblasts is known to arise in the developing limb (Bonner, 1978). Differentiated muscle cells are present within 24 hours of implantation, prior to spinal nerve formation but at a time when numerous host-derived neural crest cells can be expected to be present. Thus, it is possible that differentiated myoclusters survive for longer periods of time only when they are located in close proximity to neural crest cells and/or spinal nerve. This raises the possibility that determined myogenic precursor cells are present even in the segmental plate, but that they must mature within the somites in order to survive in the challenge environment without the aid of neural influence.

(2) an increase in the number of muscle precursor cells that have already initiated differentiation.

Undifferentiated myoD-positive cells are present in the grafted fragments of early somites (Williams and Ordahl, 1994) and it is reasonable to assume that their numbers increase with somite age. Cells already expressing MDFs in the dorso-medial somite may have no choice but to continue to differentiate, or die in the challenge environment. Many fully differentiated, post-mitotic myotome cells are present in the grafted fragments of older somites. When these fragments were labelled with BrdU, the label was incorporated in the nuclei of cells that later differentiated as muscle. Thus, the presence of BrdU labelled differentiated skeletal muscle cells suggests that the differentiated cells present at day 8 are not simply the remaining differentiated cells present in the original somite fragment, but rather, are formed by undifferentiated precursors that continue to divide in the challenge environment. It is possible that the difference in the number of differentiated skeletal muscle cells persisting until day 8 reflects the two-fold difference in the number of cells in the original transplants. However, the more than 5-fold difference in the number of muscle cells derived from early and late somites strongly suggests an increased mitotic ability of cells in the grafts from older somites.

(3) the enhanced mitotic ability of determined myogenic precursor cells in older somites.

The two behaviors seen in this assay might represent truly distinct populations of muscle cell precursors within the somite. Precedent for the idea of separate and distinct muscle precursor populations has been established by genetic methods (Patapoutian et al., 1995; Braun and Arnold, 1996; Cossu et al., 1996) and experimental embryological methods (Ordahl and Le Douarin, 1992). A recent candidate for a mitogenic agent that distinguishes early from late myotomal cells is the muscle specific FGF receptor, FREK (Marcelle et al., 1995). FREK expression is initiated in the dorso-medial somite 15 hours

after the expression of myoD, a period that corresponds closely to the timing of appearance of early and late somite muscle precursors. Thus it is possible that determined MPCs in early somites have a limited mitotic potential, while those from later somites have an enhanced mitotic potential owing to their ability to respond to ligands for FREK that are expressed in the somite (ie FGFs 4, 6 and 8) (Niswander and Martin, 1992; deLapeyriere et al., 1993; Crossley and Martin, 1995; Crossley, et al., 1996; Grass, et al., 1996). Whether these two populations are lineally related, or arise as separate populations from multipotent cells within the dorso-medial somite is unknown. Nevertheless, both are localized to the dorso-medial quadrant of the somite and are found in association with cells that have potential to form cartilage tissue. Thus, not only is the dorso-medial domain of the somite a region where important specification decisions are made for both the skeletal muscle and cartilage cell phenotypes, but different steps in skeletal muscle cell specification may also occur within it. Consistent with these observations is the recent identification of the medial lip of the dermomyotome as the site for generation of myotome precursor cells in the somite (Denetclaw, et al., 1996).

Acknowledgements

We would like to thank Anne-Gaele Borycki, Andrew Lassar, Giulio Cossu, Steve Hauschka, Olivier Pourquie, and Billie Swalla for helpful discussions at the 1995 Gordon Conference, and Christophe Marcelle for his insights on FREK. We are deeply indebted to Hal Weintraub for his seminal role in shaping the debate about transcription factors and specification. Probes from Rudi Balling (Pax1), Peter Gruss (Pax3), Bruce Paterson (CMD1) and Charlie Emerson (myf5). MF20 and QCPN antibodies were obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA. Bodo

Christ and Nicole LeDouarin each contributed immeasurably as our mentors in experimental embryological pursuits.

Figure 3.1. Surgical preparation of the notochord challenge.

Panel A shows the donor quail embryo pinned in a black Sylgard dish, ventral side facing the operator. The segmental plate and the somites used in this study are indicated.

Panel B shows a high power magnification of the quail donor somite IV, viewed from a ventral aspect, that has been cut longitudinally. The donor embryo neural tube is visible on the left side of the panel, and the intermediate mesoderm is seen to the right, rostral is at the top of the panel. The ventro-medial quadrant of somite IV has been removed via aspiration, and the dorso-medial quadrant is pictured in situ. The ventro-lateral quadrant of somite IV is also pictured.

Panel C shows a dorsal view of the chick host embryo in ovo after India ink has been injected underneath the blastoderm. The surgical field is marked.

Panel D shows the surgical field opened by application of pancreatin and use of the microscalpel; the cervical and brachial somites are exposed after reflection of the ectoderm. Host somite stage I is marked, as is the ectoderm which has been reflected with the microscalpel. Rostral is to the top of the panel.

Panel E shows the medial half of chick host somite stage I removed to accept the graft, and the quail donor fragment isolated in Panel B brought in to the surgical field.

Panel F shows the quail donor fragment tucked in place of the host somite stage I medial half.

Panel G shows the two donor chick notochords in place over the graft, extending several somites cranial and caudal to the graft region.

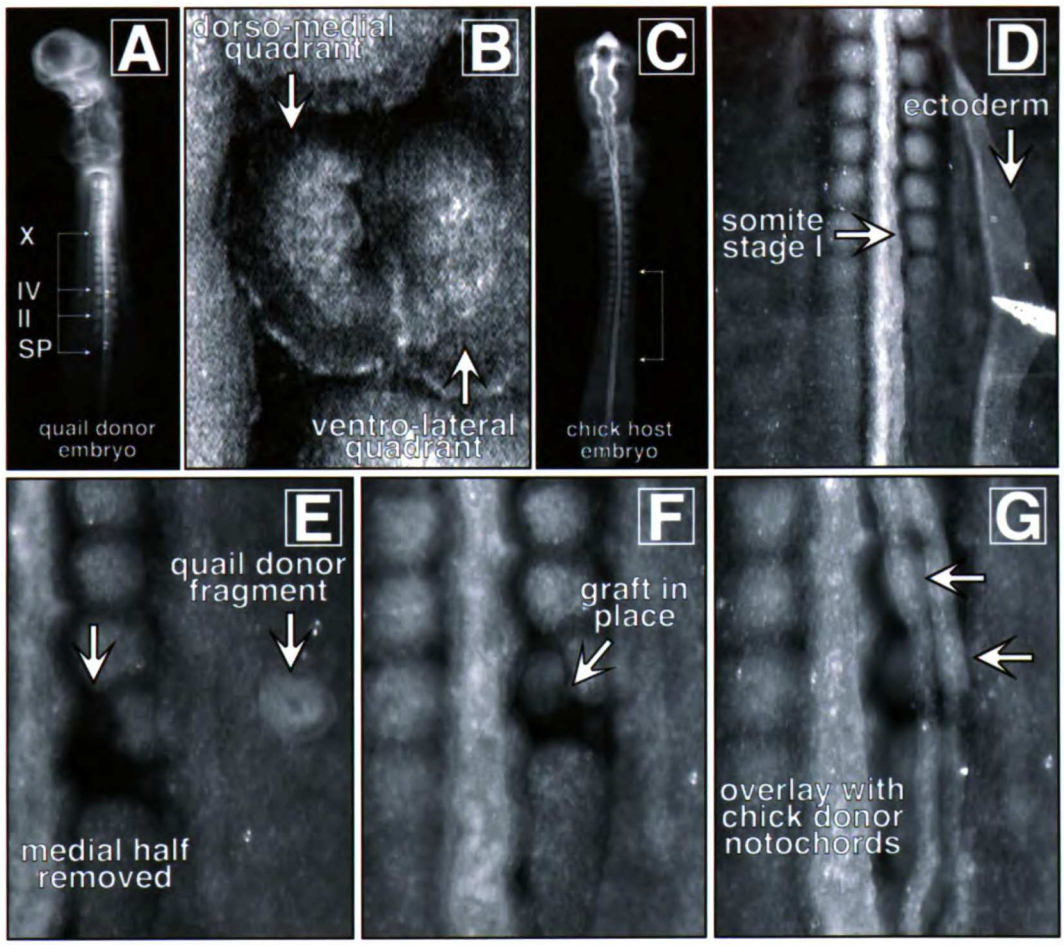
A
X
IV
II
SP
quail donor embryo
dorso-medial quadrant
B
ventro-lateral quadrant
C
chick host embryo
D
ectoderm
somite stage I
E
quail donor fragment
medial half removed
F
graft in place
G
overlay with chick donor notochords

Figure 3.1

Figure 3.2. Anatomy of the embryonic microenvironment after short and long incubation periods. Muscle precursor cells from the dorso-medial paraxial mesoderm behave differently in the dorsal and ventral epaxial domains of chimeric host embryos.

Panel A (110X) is a Feulgen stained cross section of a chimeric embryo constructed with the dorso-medial quadrant of somite stage IV showing the arrangement of tissues 5 hours post surgery. Note that the lateral half of host somite stage I is still present at the time of grafting, and that the quail donor fragment is placed next to the host notochord, in effect surrounding it with three notochords. The host notochord and chick donor notochords are marked with asterisks. The grafted quail cells have segregated into a loose mesenchyme situated next to the aortic vessel (marked), and an epithelial fragment adjacent to the ventral neural tube of the host, marked by the dotted outline.

Panel B (90X) shows the responding quail cells from a stage IV somite 24 hours post surgery. The loose mesenchyme has expanded dorsally and there is an amorphous, but contiguous group of dermomyotome-like cells in a cluster adjacent to the host notochord. Note that the host dermomyotome and myotome on the operated side of the embryo are absent in comparison with the unoperated side.

Panel C (27X) is a cross section through an 8 day chimera constructed with the dorso-medial quadrant of a stage X somite, stained with the QCPN anti-quail antibody. Quail cells are evident in both the ventral epaxial domain, and have also escaped into the dorsal epaxial domain. Note the large population of quail cells present in the vertebral cartilage model. Specimens with cells that escaped into the dorsal epaxial domain were excluded from the analysis.

Panel D (27X) is an adjacent cross section to that shown in C, stained with the MF20 anti-myosin antibody. Differentiated skeletal muscle is seen in both the dorsal and ventral epaxial domains, in the same regions that are populated by the quail cells shown in C.

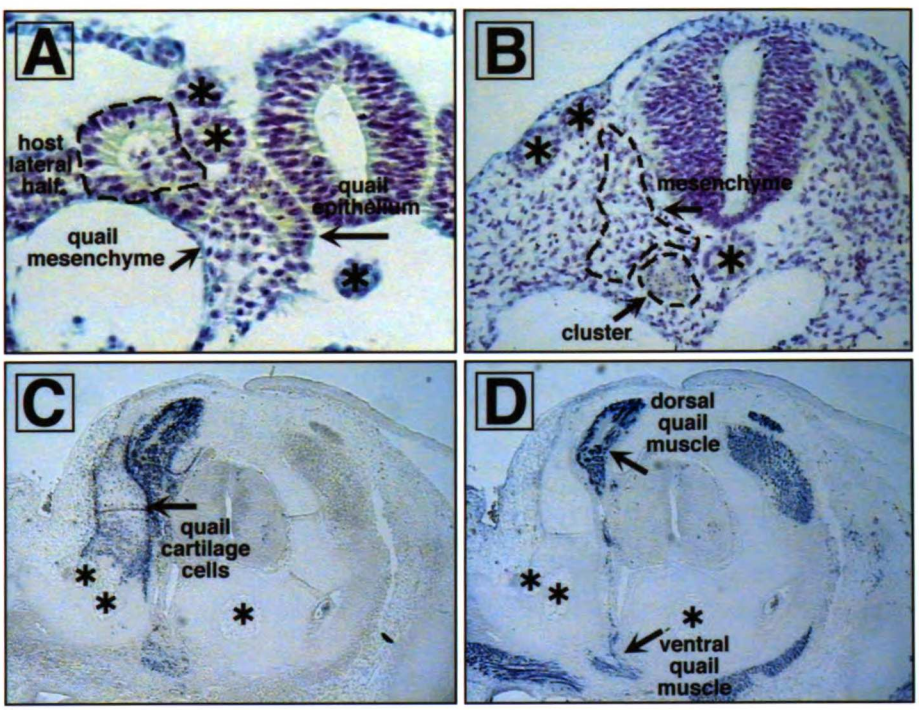
A
host lateral half
quail epithelium
quail mesenchyme
B
mesenchyme
cluster
C
quail cartilage cells
D
dorsal quail muscle
ventral quail muscle

Figure 3.2

Table 3.1. Progressively older brachial paraxial mesoderm grafts produce increasing numbers of differentiated skeletal muscle cells and consistently produce large numbers of cartilage cells in response to notochord signals.

Muscle differentiation was scored by counting quail nucleoli that appeared in MF20 positive regions of the immediately adjacent section, and was scored only in chimeras where the quail cells remained exclusively in the ventral epaxial domain. All sections in an embryo that contained visually detectable quail cells in an area that was stained with the anti-myosin antibody were included. Contribution to cartilage was assessed by histological appearance at day 8. A majority of cases from the segmental plate and from somite II produced no detectable quail muscle colonies; the few chimeric embryos that contained quail muscle cells showed only small colonies of under 20 cells total. Somite IV produced a few (3 out of 9) more robust colonies of between 100-150 cells each, although a majority of cases (5 out of 9) produced only small colonies of fewer than 20 cells. Somites X-XIII produced large numbers (greater than 200, typically thousands) of quail cells populating frank muscle in the ventral domain. Seven chimeric embryos prepared for this study contained cells that escaped confinement in the ventral domain and made large numbers of differentiated quail muscle cells in the dorsal axial domain adjacent to the neural tube.

Table 3.1

Contributions to ventral muscle and cartilage from the dorso-medial quadrant of brachial somites at different stages of maturation

		Ventral Muscle # of embryos broken down by colony size*				Cartilage # of embryos broken down by colony size*				Dorsal Escape # of embryos†
Donor Somite	n	none	small	medium	large	none	small	medium	large	n
Segmental plate	10	6	2	0	0	0	1	0	7	2
Somite II	15	7	7	0	0	0	0	0	14	1
Somite IV	11	1	5	3	0	1	0	0	8	2
Somites X-XIII	10	0	1	1	6	0	0	1	7	2

* - Small colonies are fewer than 20 cells, medium sized colonies are 100-150 cells, and large colonies are greater than 200 cells (typically thousands).

† - Dorsal Escape breakdown:

Graft	Muscle Colonies	Cartilage Colonies
Seg. Plate	1-none, 1-small	2-large
Somite II	1-none	1-large
Somite IV	1-none, 1-small	2-large
Somite XI-XIII	2-large	1-small, 1-large

Figure 3.3. Dorso-medial cells of the brachial segmental plate form cartilage and infrequently produce small clusters of nerve-dependent mononucleated myocytes in response to notochord signals.

Panel A shows a Feulgen stained cross-section (27X) of an 8 day chimeric embryo which received a graft of the dorso-medial cells from the rostral third of the segmental plate. The graft was overlaid by two additional chick notochords. The host neural tube (nt) is distorted, with the ventral region and the host notochord displaced to the unoperated side of the embryo. Both the host and donor notochords are marked with asterisks, and are visible in the ventral region of the embryo; the donor notochords are surrounded by ectopic cartilage.

Panel B shows an adjacent section stained with the MF20 anti-myosin antibody at 27X. The donor and host notochords are marked with asterisks, and the location of the host dorsal and ventral muscle masses are marked with arrows.

Panel C is a magnification at 560X showing Feulgen stained quail cells (arrows) in the mesenchyme surrounding the host vertebral cartilage model.

Panel D shows the same region of the adjacent MF20 stained section, indicating that none of these mesenchymal cells are myosin-positive. Panels E and F show two equivalent areas of the adjacent sections magnified at 560X within the cartilage model. The quail cells in Panel E (arrows) are embedded in a dense cartilage matrix, and do not stain with the MF20 antibody (Panel F).

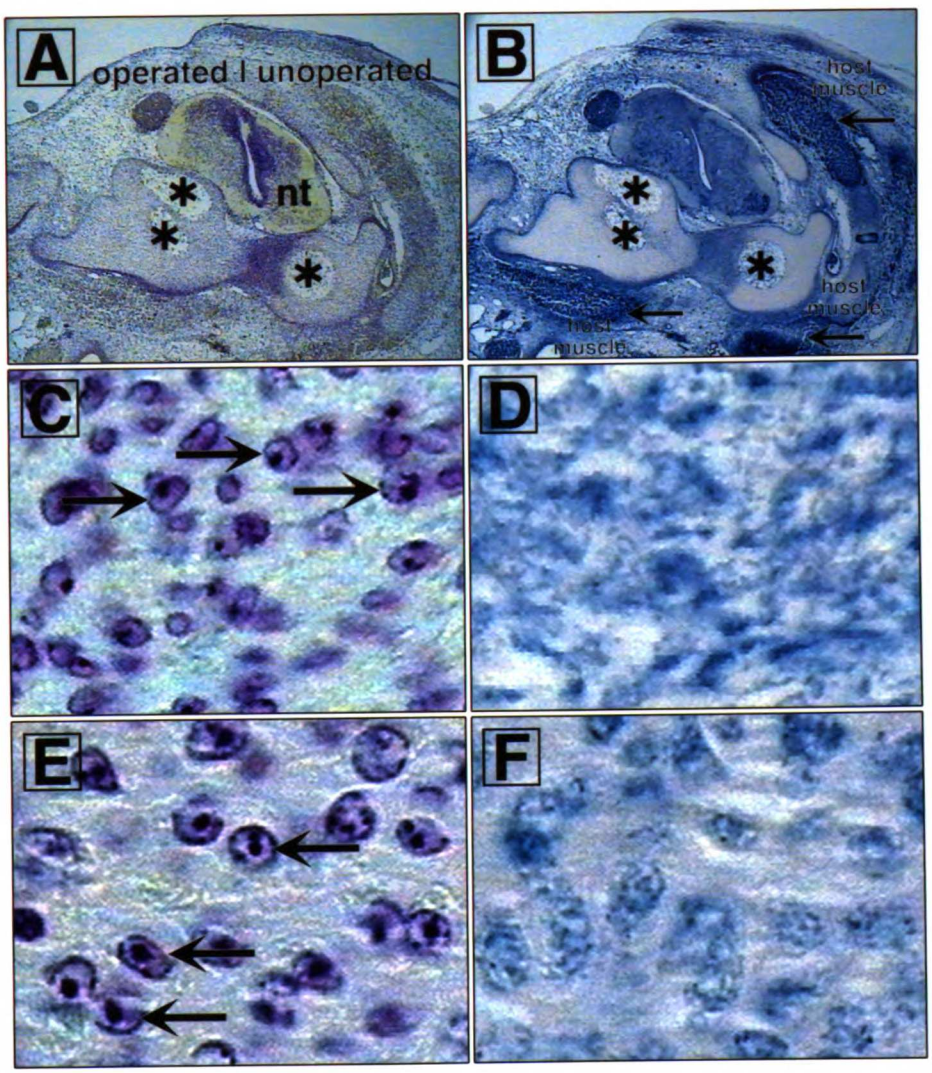
A
operated I unoperated
nt
B
host muscle
host muscle
host muscle
C
D
E
F

Figure 3.3

Figure 3.4. Dorso-medial cells from the stage II brachial somite produce cartilage and small clusters of nerve-dependent mononucleate myocytes in response to notochord signals.

Panel A (27X) shows a Feulgen stained cross-section of an 8 day chimeric embryo with a graft of the dorso-medial cells from a stage II somite. The graft was overlaid with two additional chick notochords. The same overall distorted morphology and formation of ectopic cartilage can be seen.

The MF20 stained adjacent section in Panel B (27X) demonstrates a small (fewer than 20 cells) cluster of differentiated quail skeletal muscle cells (large arrow) found in close association with the spinal nerve (marked in Panel A) which has exited the vertebral cartilage model. Host and donor notochords are marked with asterisks, and the host muscle masses in the dorsal and ventral epaxial domains are labelled with arrows.

Panel C (350X) shows a small colony of quail cells (arrows) immediately adjacent to the spinal nerve which has penetrated the vertebral model.

Panel D (350X) shows myosin expression in this small colony of quail cells (arrows).

Panels E and F (560X) are equivalent regions of the vertebral model magnified to show that the quail cells present in the cartilage model (arrows in E) are not expressing myosin (F), based on immunocytochemistry with anti-myosin.

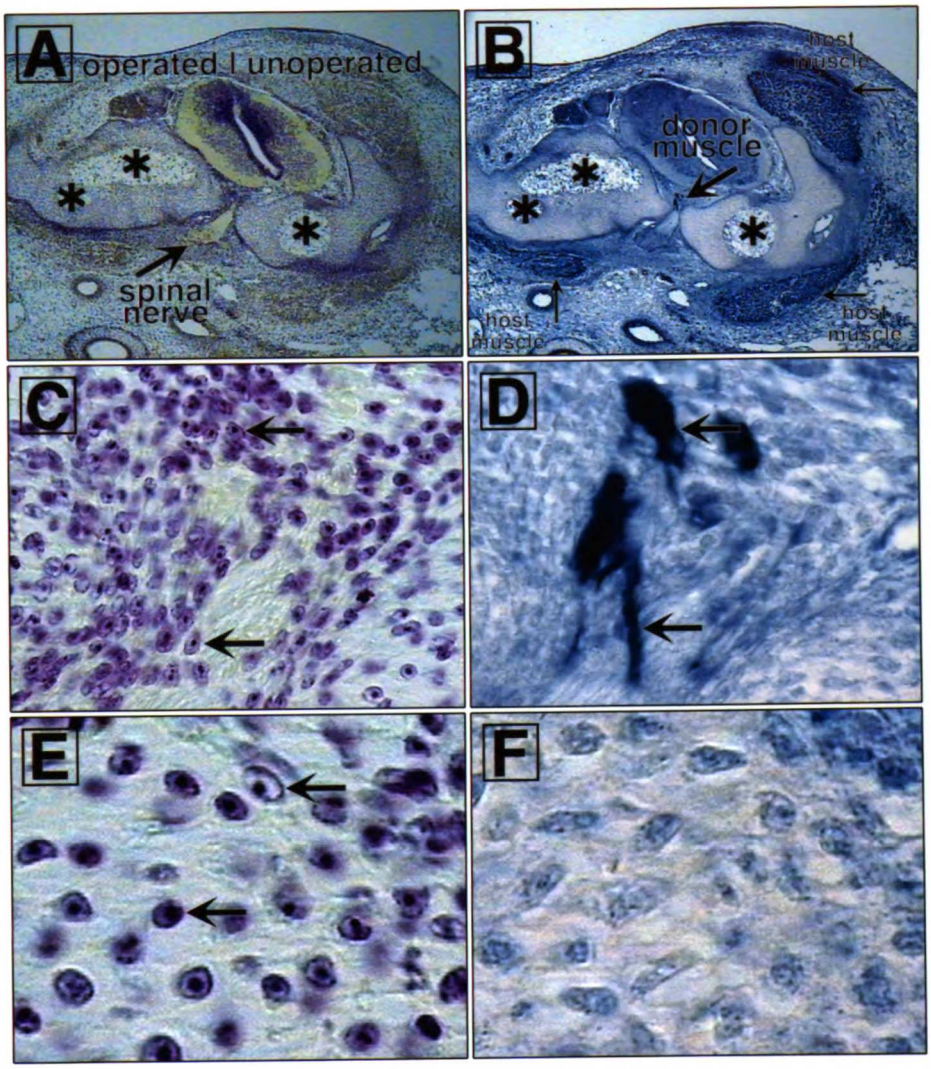
A
operated
unoperated
spinal nerve
B
donor muscle
host muscle
host muscle
C
D
E
F

Figure 3.4

Figure 3.5. Dorso-medial cells from older brachial somites (stages XI-XIII) produce differentiated skeletal muscle tissue and cartilage in response to notochord signals.

Panel A (27X) shows a Feulgen stained cross-section of an 8 day chimeric embryo which received a graft of the dorso-medial cells from a stage XI somite, which was overlaid with two additional chick notochords. The same distorted morphology and ectopic cartilage surrounding the donor notochords is evident. Host and donor notochords are marked with asterisks.

Panel B (27X) shows the adjacent section stained with the MF20 antibody identifying a large block of differentiated quail skeletal muscle cells (large arrow) ventral to the vertebral cartilage model that is not associated with spinal nerve. Host muscle domains in the ventral and dorsal epaxial compartments are labelled.

Panel C (560X) is a magnification of the large block of differentiated quail skeletal muscle stained with the Feulgen technique. The quail nucleoli are clearly visible (arrows), fused into elongated myotubes, which stain positive with the MF20 antibody (arrows) in Panel D (560X).

Panels E and F (560X) are equivalent areas of the cartilage model in the cross sections showing again that the quail cartilage cells (arrows) do not express myosin, as judged by lack of staining with MF20.

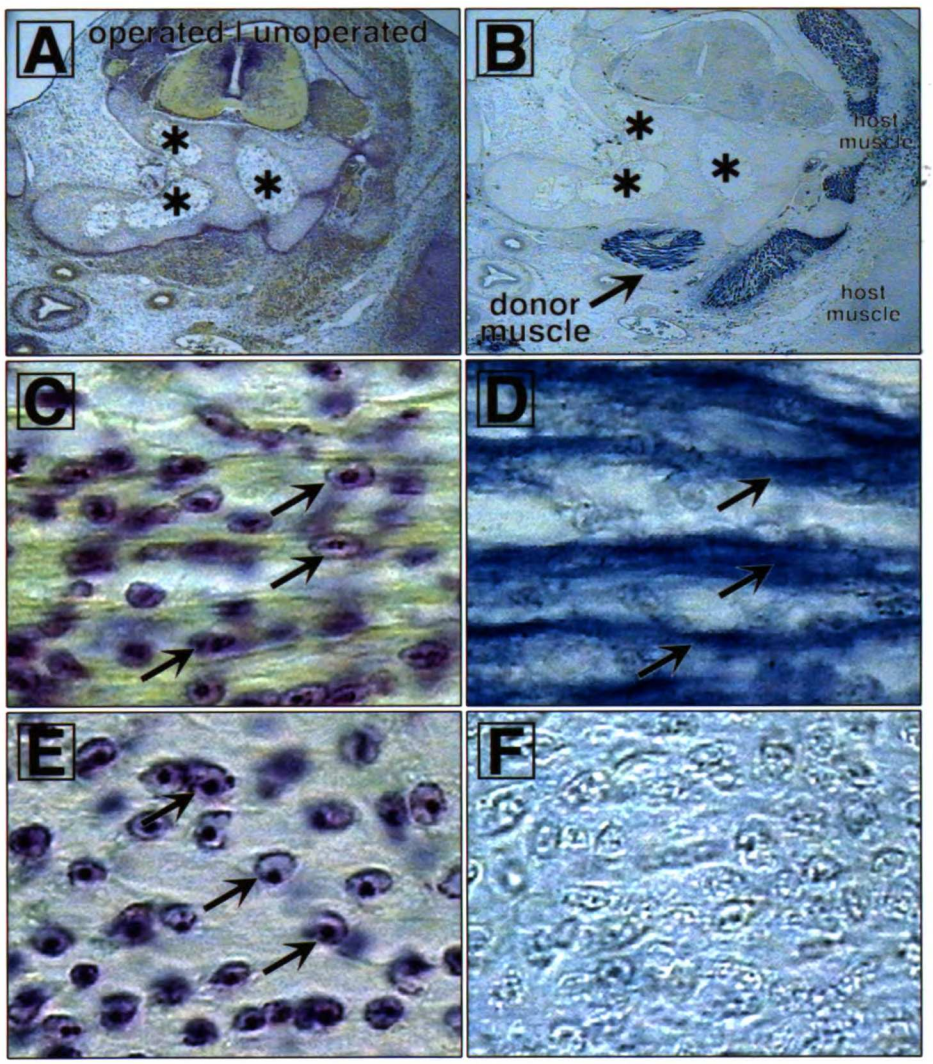
A
operated
unoperated
B
host muscle
donor muscle
host muscle
C
D
E
F

Figure 3.5

Figure 3.6. Undifferentiated graft-derived cells resident in the mesenchyme surrounding the cartilage model do not express mRNAs characteristic of muscle precursor cells.

Panel A (56X) is a Feulgen stained cross-section of an 8 day chimeric embryo which received a graft of dorso-medial cells from the stage IV brachial somite, overlaid with two additional chick notochords. The host notochord is marked with an asterisk.

The boxed region in Panel A is magnified in Panel B (350X), demonstrating the nucleoli of quail cells that lie outside the cartilage model in mesenchyme (arrows).

Panel C (56X) is an adjacent section stained with the MF20 antibody and demonstrates no antibody localization in the boxed region.

Panel D (56X) is an adjacent section hybridized to myoD and exposed for two weeks, again showing no localization of this marker for undifferentiated skeletal muscle cells.

Panel E (56X), a three week exposure, indicates the same result with a probe for the myf5 mRNA, and Panel F (56X), also a three week exposure, shows that this region is also Pax3 negative.

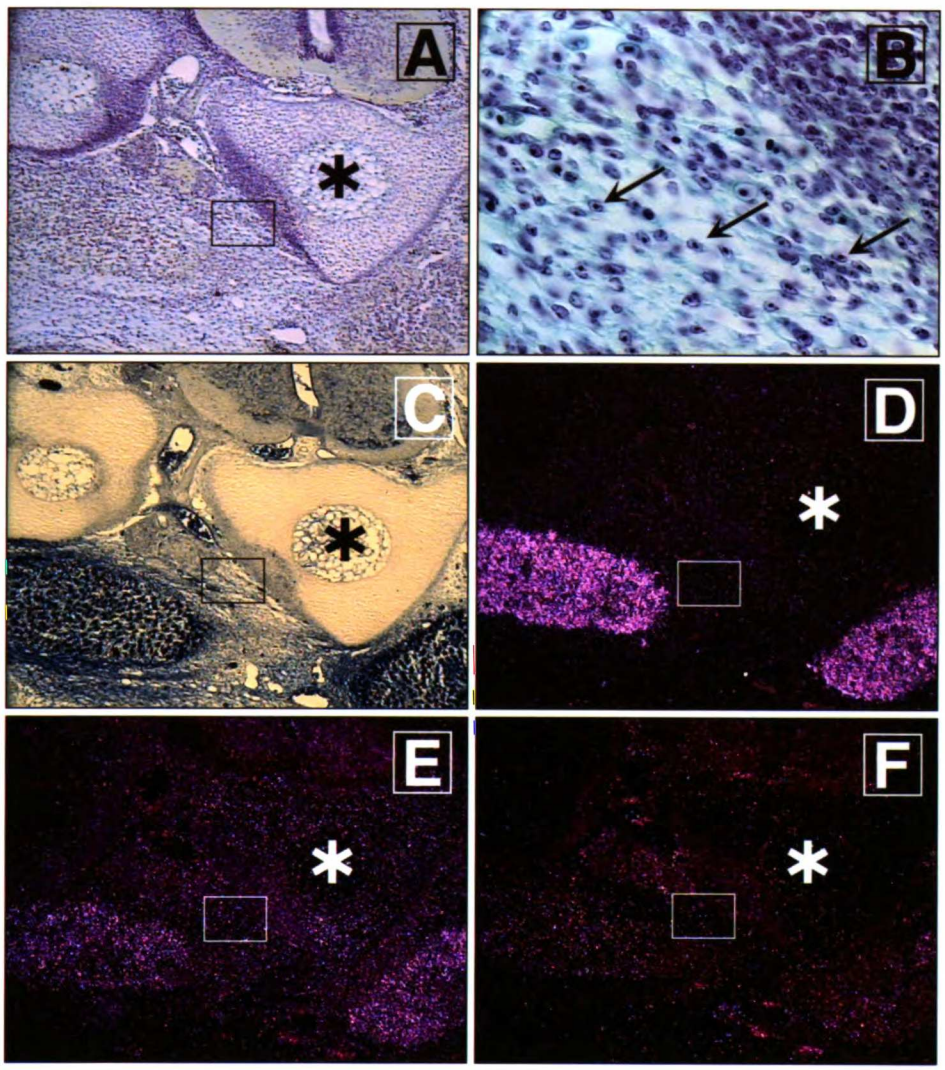

Figure 3.6

Figure 3.7. Rapid differentiation of muscle precursor cells can occur in close proximity to grafted notochords.

Panel A (56X) shows a Feulgen stained cross-section of a chimeric embryo harvested 24 hours after surgery. The host received a graft of the dorso-medial cells from a stage XI brachial somite overlaid with two notochords. The quail cells are surrounded by the thin dotted line. Host and donor notochords are marked with asterisks. A large mass of contiguous quail cells is present immediately ventral to the two donor notochords; a few loose quail mesenchymal cells are seen ventral to and surrounding this mass.

Panel B (56X) shows an adjacent section stained with the MF20 antibody. Note that the contiguous mass is differentiated muscle cells, and that the myotome on the unoperated side of the chimera is also MF20 positive.

Panel C (56X) shows an adjacent section hybridized to the quail myf5 probe, indicating hybridization in the differentiated muscle cell regions seen in Panel B.

Panel D (56X) shows an adjacent section hybridized to myoD, again showing strong expression in the regions of muscle differentiation.

Panel E (56X) is an adjacent section hybridized to Pax3, showing that the implantation of donor chick notochords has effectively abolished Pax3 expression in cells of the operated paraxial mesoderm, and has extinguished some expression in the dorsal neural tube on this side of the embryo as well.

Panel F (56X) is an adjacent section hybridized to Pax1, showing expression in the mesenchymal cells on both sides of the chimera, with some enhancement of expression seen immediately adjacent to the muscle domains on the operated side of the embryo.

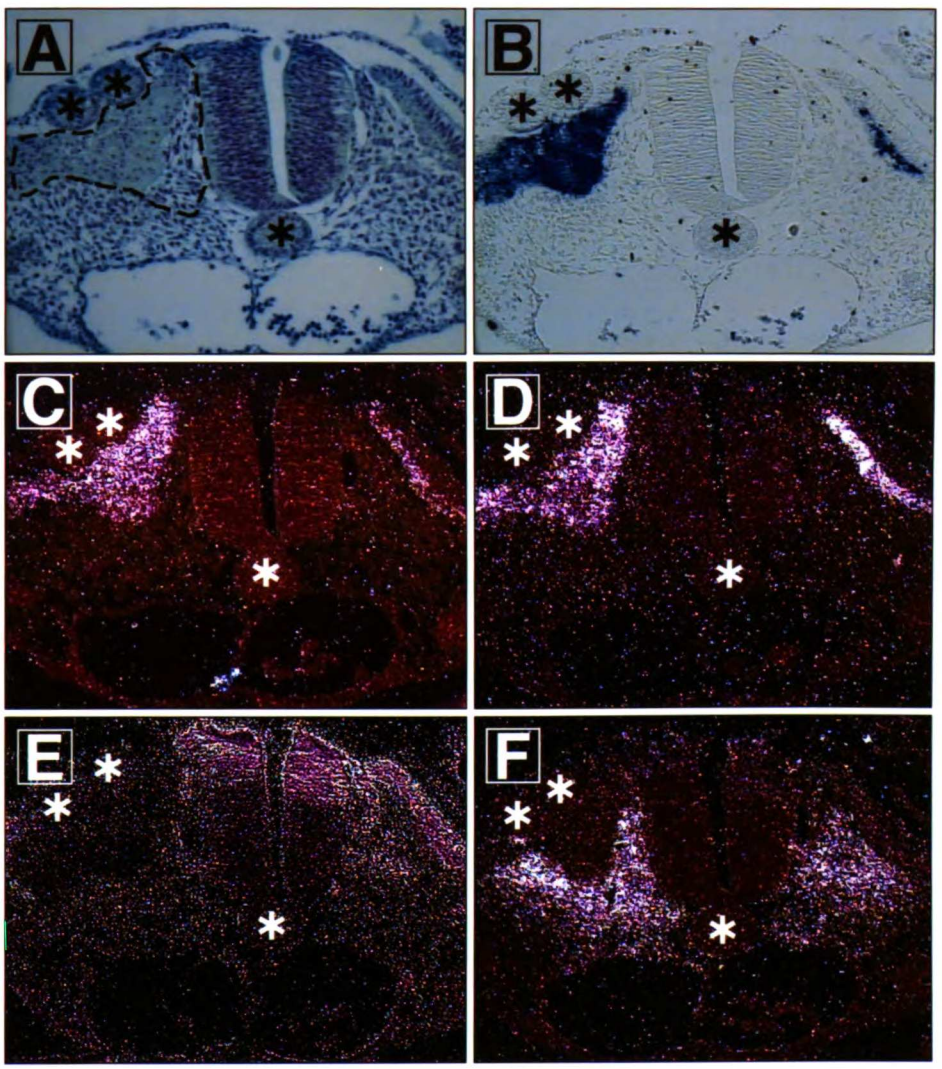

Figure 3.7

Figure 3.8. Surgical removal of cells fated to form sclerotome from the ventro-medial regions of donor somites. Older somite grafts (XI-XIII) contain differentiated skeletal muscle cells.

Panel A is a Feulgen stained cross-section through a stage II quail somite immediately after removal of the ventro-medial quadrant. After the ventro-medial cells have been removed, the intact dorsal somite epithelium remains. The arrow is placed at the position where the medial epithelium is separated from the lateral epithelium.

Panel B is a Feulgen stained cross-section through a stage IV somite after removal of the ventro-medial quadrant. Both the somitocoel and sclerotome cells are cleanly removed from the dorso-medial somite epithelium. The arrow is at the location of the incision to separate medial from lateral half.

Panel C is a Feulgen stained cross-section through a stage XI donor somite after sclerotome excavation. The arrow indicates the location of the incision separating medial from lateral halves.

Panel D is an adjacent section to that shown in Panel C, stained with MF20, demonstrating the presence of already differentiated skeletal muscle cells in the fragment at the time of grafting (arrowhead).

Figure 3.9. Mitotically active muscle precursor cells differentiate rapidly in response to notochord signals.

Panel A (56X) shows a cross-section of a 3 day chimeric embryo 24 hours after grafting a stage XI dorso-medial fragment that was labelled with BrdU for 90 minutes prior to grafting into the notochord challenge. This section is stained with the MF20 antibody, revealing differentiation of skeletal muscle tissue; the boxed area is magnified in Panels B-D.

Panel B (350X) shows the region containing differentiated skeletal muscle tissue, as identified by the MF20 antibody.

Panel C (350X) is an adjacent section showing the location of quail cells in the grafted region by identification with the QCPN anti-quail antibody.

Panel D (350X) is another adjacent section showing that many of the nuclei in the grafted tissue that differentiated as muscle are labelled with BrdU, as detected by the IU-4 anti-BrdU antibody.

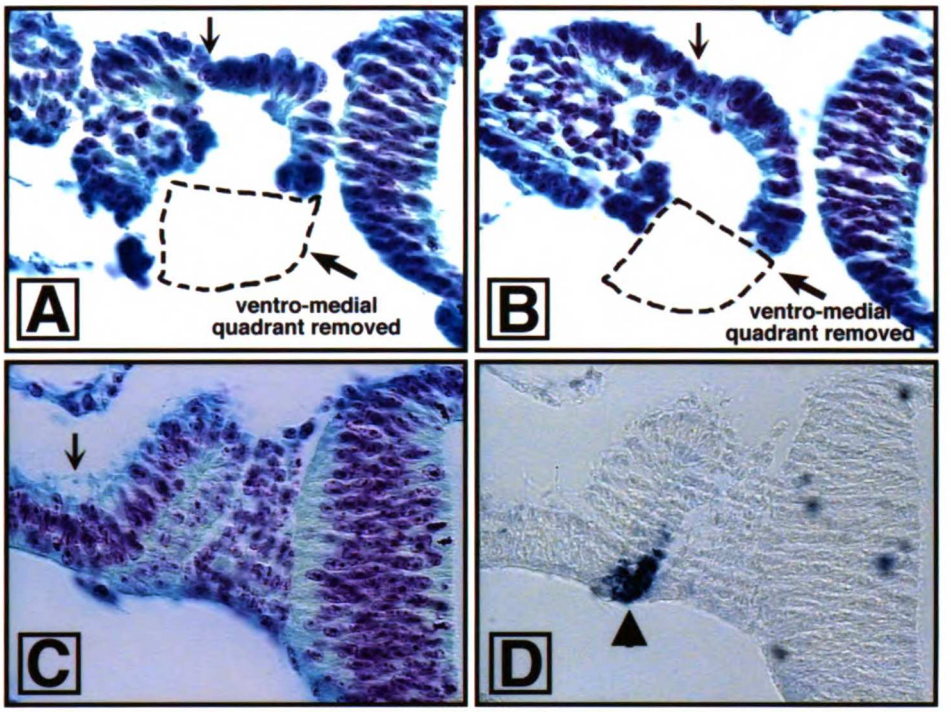

Figure 3.8

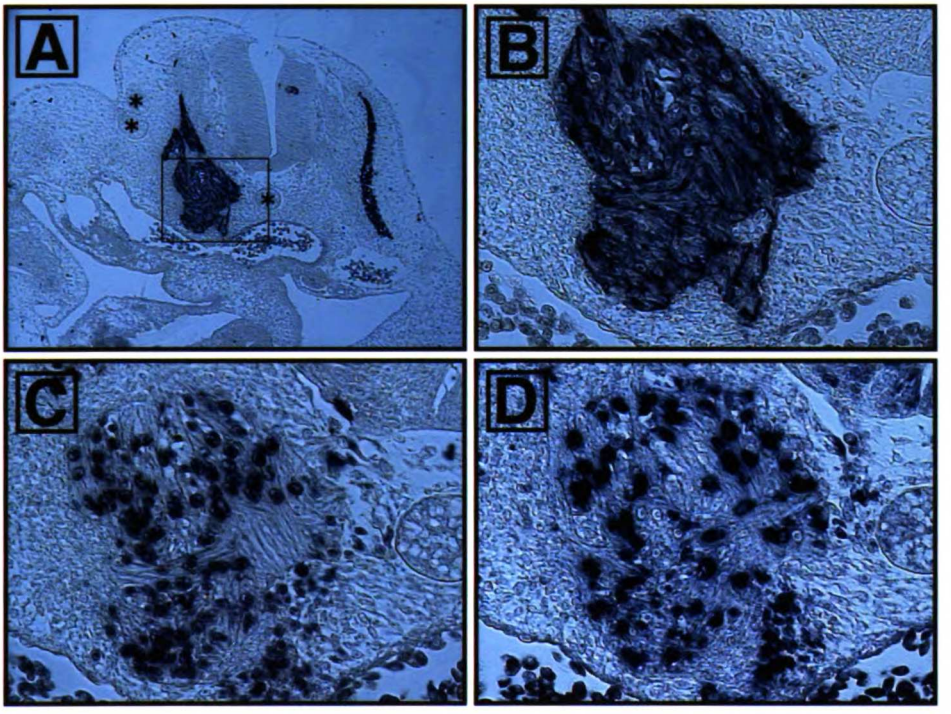

Figure 3.9

Figure 3.10. Summary of muscle precursor cell properties

Myogenic precursor cells from the segmental plate. Dorso-medial segmental plate rarely forms differentiated myoclusters of fewer than 20 cells, and is converted almost exclusively to cartilage. The paired-box transcription factor, Pax3, is expressed throughout this tissue, at levels detectable by in situ hybridization. Myf5 expression can only be detected by PCR amplification, and myoD is not expressed in this tissue .

Myogenic precursor cells from the stage II somite. Dorso-medial cells of the stage II somite form myoclusters only 50% of the time, and are converted completely to cartilage in the remaining cases. In situ detectable myoD expression begins in the dorso-medial epithelium of the stage II somite; at this time, Pax3 expression is extinguished in these cells.

Myogenic precursor cells from the stage IV somite. Dorso-medial cells of the stage IV somite often form small myoclusters and occcasionally form large numbers of differentiated, disorganized muscle cells. Cartilage is always elicited from these grafts. MyoD expression is still detectable, and the onset of in situ detectable myf5 expression is seen.

Myogenic precursor cells from older somites (XI-XIII). Dorso-medial cells from older somites have acquired an enhanced mitotic potential that results in the consistent formation of large numbers of skeletal muscle cells organized into recognizable muscle tissue. In situ detectable expression of myoD, myf5 and myogenin is seen, as well as the presence of fully differentiated myotome fibers expressing myosin heavy chain. The expression of Pax3 is restricted primarily to the lateral border of the somite.

Myogenic commitment in the dorso-medial paraxial mesoderm

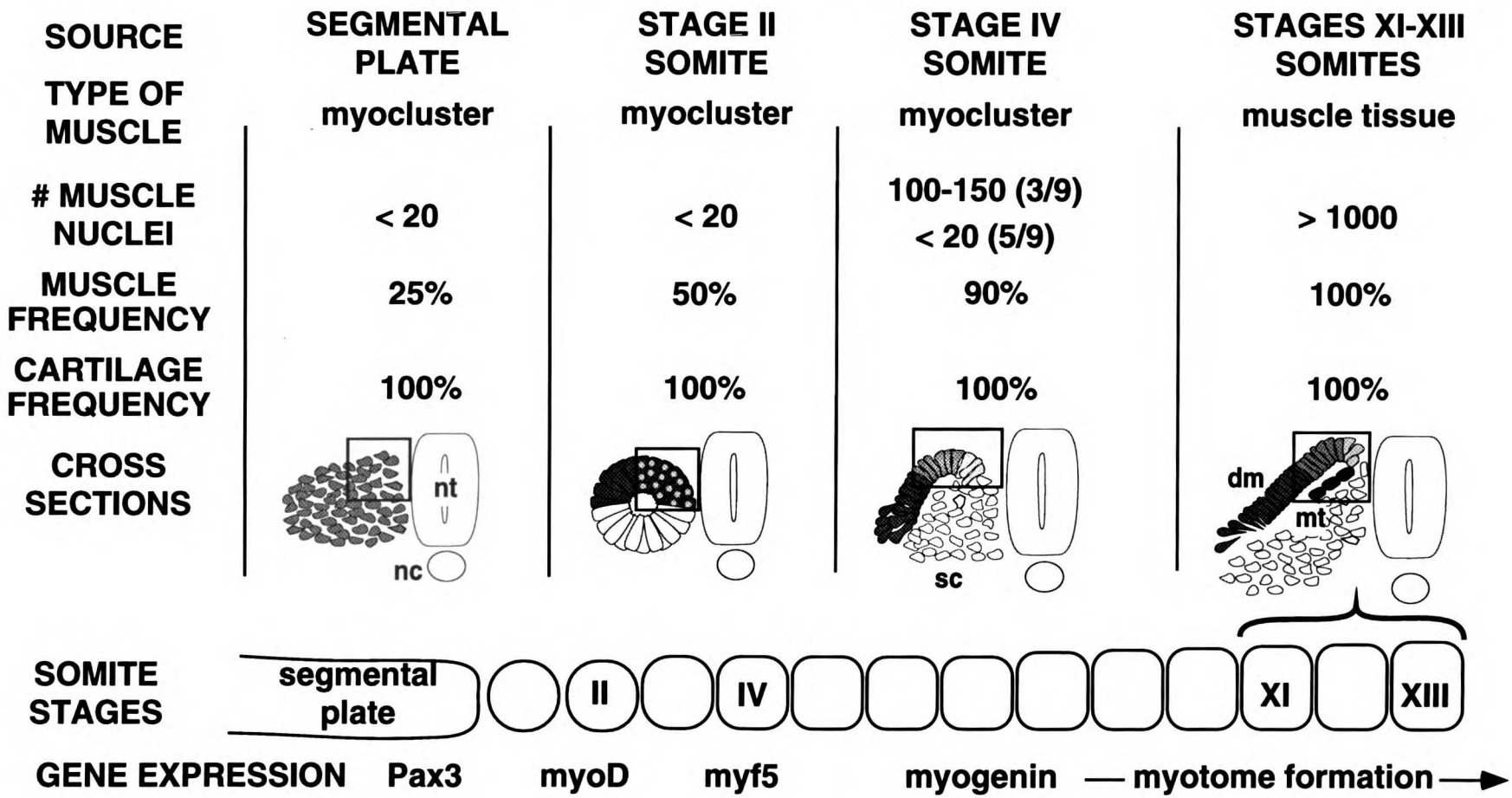

Figure 3.10

CHAPTER FOUR

Determination of muscle precursor cells in the avian limb bud precedes overt expression of myogenic factors

Abstract

Previous studies combined in situ hybridization with experimental embryology techniques to identify expression of Pax3 mRNA as a marker for muscle precursor cells that is expressed in the limb bud prior to the expression of myogenic bHLH factors. We have employed an in vivo bioassay to analyze whether cells expressing the Pax3 marker gene are determined to form skeletal muscle, or whether they are in an early stage of cell-type specification that still allows the expression of other mesodermal cell fates. When muscle precursor cells are still present in the lateral half of the epithelial somite, they are susceptible to the cartilage inducing properties of the notochord and produce large numbers of cartilage cells that are recruited into chimeric cartilage models with host derived cells. However, a small number of cells present in the lateral half of the epithelial somite can differentiate as muscle even when trapped within the tissue of the formed cartilage model. When lateral half somite cells migrate into the limb bud as individual mesenchyme cells, they continue to express the Pax3 mRNA, but are now no longer able to respond to cartilage inducing signals from the notochord and instead produce only skeletal muscle. Thus, we have defined two new steps in the specification of skeletal muscle. Pax3 expressing cells of the paraxial mesoderm, while still entrained within the epithelium of the somite are in an early stage of specification that is susceptible to cartilage inducing signals from the notochord. When these cells begin to migrate into the limb field as mesenchyme, they progress into a new stage of specification and behave as determined muscle precursor cells. Thus, expression of Pax3 and migration into the limb field, are two consequent steps correlated to the determination of myogenic precursor cells in the embryonic limb bud. The two day period of presence in the limb prior to muscle differentiation will provide a useful

experimental system for studying the molecular mechanism of specification of limb muscle precursor cells.

Introduction

The discovery of myogenic basic helix-loop-helix (bHLH) factors as transcription factors that control contractile gene expression was predicated on the assumption that a simple gene regulatory mechanism was responsible for the coordinated expression of contractile proteins during skeletal muscle differentiation (Devlin and Emerson, 1978; Devlin and Emerson, 1979; Lassar, et al., 1986; Pinney, et al., 1988). The notion that "master control genes" are involved at "nodal points" in the gene transcription pathway that drives embryonic development continues to gain currency, as factors that result in formation of ectopic eyes (Halder et al., 1995) and limbs (Cohn et al., 1995) have been isolated.

The myogenic bHLH factors are expressed in the somites and limb buds of vertebrate embryos immediately prior to the expression of contractile protein genes. This profile of expression is consistent with what is known about their function in the regulation of contractile protein gene expression. Recently, it was shown that Pax3, a paired domain transcription factor, is expressed in cells of the somite and migratory muscle precursors to limb muscle, well in advance of the expression of the myogenic regulatory factors (Bober, et al., 1994b; Goulding, et al., 1994; Williams and Ordahl, 1994). Mutations in the Pax3 gene prevent the formation of muscle in the limbs, and cause aberrations in the formation of myotomal muscles (Franz, et al., 1993). However, Pax3 is not believed to function directly in the specification of skeletal muscle cells, as lateral half somite cells from Pax3 mutant mice differentiate as skeletal muscle when grafted into the limb bud of developing chick embryos (Daston et al., 1996). Pax3 is believed to function in regulating cell motility by regulating expression of the c-met receptor for scatter factor (Epstein et al., 1996). This

conclusion has been supported by experimental disruption of the c-met gene, which also causes a severe deficit in the formation of limb musculature (Bladt et al., 1995).

Stages in specification of embryonic cells fated to form a specific cell type are defined experimentally (Slack, 1983). We have recently described an assay for the description of stages in the specification of myotomal precursor cells of the medial somite, using ectopic notochord grafts as a means of "challenging" skeletal muscle precursor cells to differentiate as cartilage. We have employed this assay to determine whether or not the migrating skeletal muscle precursor cells of the chick limb bud can form only skeletal muscle and have restricted their options to choose other mesodermal fates. This assay was performed during the two day window of time when muscle precursor cells are present in the limb bud expressing Pax3 mRNA, prior to the overt expression of the myogenic basic helix-loop-helix transcriptional regulators. We have found that migratory Pax3 expressing cells are incapable of responding to cartilage-inducing signals from the notochord, and produce only skeletal muscle. The cell biological basis of this behavior is discussed, and a possible role for paired-domain transcription factors in the specification of the skeletal muscle lineage is considered.

Methods

Embryo culture and surgery

Fertile chick eggs were obtained from Western Scientific Products (Sacramento, CA); fertile quail eggs were obtained from Strickland Farms (Pooler, GA). Eggs were incubated in a humidified, forced draft incubator at 37.6 C for approximately 48 hours until stage 12HH (Hamburger and Hamilton, 1951; Hamburger and Hamilton, 1992). Procedures for preparing the host embryos for surgery have been published elsewhere (Ordahl and Christ, 1996; Williams and Ordahl, 1996).

Grafts of the dorso-lateral half of the stage II somite were prepared as described in Chapter 3, except that the ventro-lateral portion of the somite was removed by aspiration in order to access the dorso-lateral half. Grafting supernumerary notochords was performed exactly as described in Chapter 3. The chimeric host embryos were returned to the incubator and processed for histological examination as described below.

Chimeric limb buds for step 1 of the surgical procedure were produced as described in Chapter 1. This procedure is briefly illustrated in Figure 4.1. A stage 12HH chick host is prepared, and an incision made in the ectoderm just lateral to the neural tube at the level of the segmental plate (Panel A). With the segmental plate now accessible to surgery, an incision is made at the lateral margin of the segmental plate medial to the intermediate mesoderm, the segmental plate is macerated by additional strokes with the scalpel, and the fragments aspirated with pipette (Panel B). Panel C shows a quail segmental plate that has been removed from a donor embryo; carbon particles mark the rostral tip. This donor tissue is then grafted into place in the host embryo (Panel D); the ectoderm is replaced and the egg returned to the incubator for 48 hours. Panel E shows the stage 22 chimeric embryo. Segmentation at the brachial level of the axis is undisturbed by the operation, and normal outgrowth of the limb bud on the operated side of the chimeric animal is seen. The chimeric limb bud (Panel F) is stained with the vital dye, Nile blue, to stain the ectoderm and show that other structures like the apical ectodermal ridge (AER) are intact.

Figure 4.2 shows step 2 of the procedure for grafting chimeric limb bud cells from the stage 22 chimeric donor into a new stage 12HH host. Panel A shows the chimeric limb bud, with ectoderm scored in the mid-dorsal line and around the AER. This limb bud while still attached to the chimeric donor, is incubated for 30 minutes at 37 C in Tyrode's solution (Sigma T2145), after 25 ul of a 1% solution of collagenase has been applied topically. The ectoderm is then peeled away, and incisions made to separate the dorsal and ventral quail muscle masses from the chick chondrogenic core of the limb (Panel B). In

some cases, whole tissue fragments of the dorsal or ventral muscle masses were grafted into a new stage 12HH host embryo next to the host notochord in place of somites I and II. After 6 days of incubation, sections of the chimeric embryos were examined to identify the regions populated by quail cells.

In other experiments, the chimeric limb cells were manipulated prior to engraftment into the new chick host. Panel C shows the quail dorsal muscle mass stripped of chick ectoderm and separated from the rest of the limb bud; it is now incubated in 0.25% trypsin for 30 minutes at 37 C. The quail limb mass is then moved to a solution of 10% fetal calf serum, and the cells are drawn into a mouth operated micropipette, and dispersed to monocellularity (Panel D). In some cases presented in this chapter, the dissociated limb cells were incubated in a solution of 10 ug/ml cytochalasin D (Fluka 30385) in DMSO (Fluka 41640) for an additional 30 minutes at 37 C. After being drawn into a micro-loader tip in an ultra pipette (Eppendorf 5242-956003), the quail cells are loaded into the back end of a needle drawn from a microcapillary (Panel E). The needle is placed inside a conical centrifuge tube and spun for 5 minutes at 3000 rpm in a table top centrifuge. The needle is then attached to surgical tubing equipped with a mouthpiece, and the needle tip broken off. The cells can be extruded from the microcapillary to form an organoid that is roughly the shape and size of the segmental plate (Panel F). In panel G, a stage 12HH chick host embryo has been prepared as described in Figure 1, and in Panel H the organoid composed of marked quail pre-muscle cells (with a small admixture of chick limb mesenchyme cells) is grafted in place of the host segmental plate. In some cases presented in this chapter, two additional notochords are grafted over the top of the dissociated chimeric limb cells (not shown). Panel I shows the ectoderm replaced. The host is then returned to the incubator for 6 days, and the chimeric embryo is harvested for histological examination.

Histology and immunohistochemistry

After 6 days of incubation, the chimeric embryos are removed from the egg into a bath Tyrode's solution, trimmed free of extra-embryonic tissues and fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for approximately 3 hours. The embryos are dehydrated through 100% ethanol, cleared in xylene for 1.5 hours and infiltrated and embedded in paraffin wax for sectioning. Sections were taken at 7um on a rotary microtome. Adjacent sections were placed on different slides for either Feulgen staining or immunohistochemistry.

Antibody staining was performed with the MF20 monoclonal supernatant provided by The Developmental Studies Hybridoma Bank at the University of Iowa. Briefly, the sections were dewaxed and dehydrated through an ethanol gradient, then incubated in water followed by PBS. After blocking for 20 minutes at room temperature in 3% normal horse serum in PBS, the MF20 supernatant was applied at a dilution of 1:100 in blocking serum for 30 minutes at room temperature. Sections were then rinsed in PBS for 10 minutes and biotinylated horse anti-mouse serum applied according to the manufacturers instructions (Vectastain alkaline phosphatase anti-mouse IgG kit AK5002). After application of ABC complex , the slides were rinsed in Genius system Buffer 1 (Boehringer Mannheim) twice for 15 minutes each, and then once in Buffer 3. NBT/BCIP development was performed according to the manufacturers instructions (Promega). The slides were rinsed for 2 minutes in water and then dehydrated, cleared and mounted in Accumount (Scientific Products). Adjacent sections were examined on the Zeiss Axiophot to score quail cells in either muscle tissue or in differentiated cartilage tissue (Tables 4.1 and 4.2). Images were collected on the Optronix CCD camera (DEI 470), and processed in Adobe Photoshop 3.0 for printing on a Fujix Pictrography 3000 printer.

Results

Response of dorso-lateral somite cells to ectopic notochord grafts

To follow the process of specification of limb muscle precursor cells from their origin in the paraxial mesoderm, we grafted dorso-lateral fragments of the stage II somite into the notochord challenge. Figure 4.3 shows the arrangement of tissues and cells in this environment. Panel A shows the large ectopic cartilage bulge in the vertebral cartilage model on the operated side of the host, indicating that the grafted notochords retained cartilage inducing potential. Panel B shows an adjacent section stained with MF20, indicating the repression of muscle differentiation in the dorsal epaxial domain on the unoperated side of the embryo, in response to the notochord signals. The appearance of host muscle in the ventral epaxial domain varies from embryo to embryo. This muscle is believed to form from retro-migration of limb muscle precursors into the axial domain. The boxed area in Panel A is magnified in Panel C, showing the prominent quail nucleoli distributed in the cartilage model. The boxed area in B is shown in Panel D, confirming that no expression of the myosin heavy chain molecule is seen in these cells. In all examples, greater than 99% of graft derived quail cells populate both the normal vertebral body surrounding the host notochord, and the ectopic cartilage induced by the supernumerary notochords.

In 3 of 5 cases where dorso-lateral quadrants from stage II quail brachial somites were grafted into the notochord challenge (Table 4.1), small numbers of quail cells that differentiate as skeletal muscle can be seen. Figure 4.4, Panels A and B show a different embryo with ectopic cartilage and repression of host dorsal skeletal muscle on the operated side of the embryo, as seen in the previous figure. The boxed area in Panel A shown in Panel C depicts a typical case in which a few quail cells are positioned immediately adjacent to the intervertebral foramen, and have differentiated as skeletal muscle. The location of

these cells adjacent to the exit for the spinal nerve suggests a requirement for nerve associated factors for skeletal muscle differentiation. These cells are similar in appearance to the small nerve-dependent myoclusters produced from younger dorso-medial somites in the notochord challenge (Chapter 3).

In 1 of 5 cases, quail cells were seen to differentiate as skeletal myocytes deep within the cartilage model. Figure 4.5.1 shows a small colony of high-level myosin expressing cells located in the cartilage model. Panels A and B show the ectopic cartilage formed on the operated side of the embryo in response to the grafted notochords and the repression of muscle formation in the dorsal and ventral domains. The boxed area in Panel A is magnified in Panel C, showing a small cluster of quail nucleoli located deep within the cartilage model, that appear bipolar and are located adjacent to a blood vessel. Myosin expression in these cells is very strong, and the cells have more of the morphological appearance of true skeletal muscle cells (Panel D). Approximately 60 cells comprise this colony.

Figure 4.5.2 shows an example of low-level myosin expression in a clone of graft derived muscle cells differentiated deep within the cartilage matrix. Panels A and B indicate the overall morphology of the graft environment, as above. Here the quail cells located in the cartilage model (Panel C) have a morphology indistinguishable from the surrounding differentiated cartilage cells. They are located a significant distance from the dorsal neural tube and overlying ectoderm. The boxed area in Panel A shown in panel D indicates that these quail cells react with the anti-myosin antibody, MF20. The cells are not arranged in the form of bipolar, multinucleate myotubes. Approximately 80 cells comprise this colony.

Grafting dissociated chimeric limb cells into the notochord challenge environment

Somite derived cells present in the avian limb are fated almost exclusively to skeletal muscle, with a minor contribution to vascular endothelium (von Kirschhofer, et al., 1994). During their migration, we have previously shown that they express Pax3 mRNA at high levels (Williams and Ordahl, 1994), from stage 18HH onward. The onset of myogenic factor expression does not occur until stage 24HH, and is followed almost immediatedly by overt skeletal muscle differentiation. To establish whether the migrating myogenic precursor cells of the limb bud are determined to form only skeletal muscle at a time when they express Pax3 but do not yet express myogenic factor mRNAs (stages 20-22HH), we challenged these cells with cartilage inducing signals from the notochord. To insure that any behavior seen was not a result of specific interactions with the mesenchymal cells of the limb, we dissociated them from their contacts with surrounding cells and matrix. In 8 of 9 cases (Table 4.2), no quail cells were identified in the cartilage model surrounding the neural tube and notochord, and the quail cells were distributed entirely in muscle tissue.

In tissue sections from the center of the grafted limb cells, there was often no identifiable neural arch, and an incomplete vertebral body on the operated side of the embryo. The myotome on the operated side of the embryo was often smaller than the myotome on the unoperated side, and was often misshapen. However, many thousands of quail cells were arranged in histologically distinct muscle tissue parallel to the main body axis of the embryo. This muscle tissue was found in both the dorsal and ventral epaxial domains, and was often located immediately adjacent to well formed cartilage models (described below). Figure 4.6 shows a representative section; Panels A and B show the arrangement of supernumerary notochords and the presence of graft derived dorsal and ventral muscle on the operated side of the embryo. The boxed area in Panel A in the ventral region nearest the maximal notochord influence is magnified in Panel C. The quail cells in

this section are present in myotubes that are cut in cross-section. Panel D shows the boxed area in Panel B magnified, demonstrating the strong expression of myosin in the quail cells in Panel C.

Cartilage models were morphologically complete in sections at the ends of the grafted tissue. Well formed lamina, pedicles, neural arch and vertebral body were in evidence. The host cells that would normally be expected to form these vertebrae were removed prior to grafting. Therefore, the origin of the chick cells that formed the vertebral models is unknown. Resegmentation of the sclerotome cells from the remaining somites cranial and caudal to the graft may have enabled the formation of these vertebrae. A single case (1 of 9, Table 4.2) showed the presence of quail cells in the cartilage model. These cells were counted manually in serial sections. The total number of quail cells in the entire graft was then estimated by counting the quail cells in all tissues of a representative section, and multiplying by the number of sections. The percentage of quail cells that differentiated as cartilage was always below 0.5% of the total number of quail cells descended from the original graft. These few cells were able to join the host cells in formation of a chimeric cartilage structure, but never formed an isolated cartilage tissue independent of host cells. As shown in Figure 4.7, the cells in the cartilage model behave in two ways. Panels A and B show the distribution of tissues in the notochord challenge. The boxed area labelled C shows that quail cells can be found in differentiated cartilage tissue, and in the perichondrial sheath surrounding the cartilage model. Panel D confirms that these cells do not express myosin heavy chain. The boxed area labelled E shows a small population of quail cells that are morphologically indistinguishable from true cartilage cells. The adjacent section in Panel F reveals that they are weakly expressing myosin heavy chain, as was seen in Figure 4.5.1.

Dissociated chimeric limb bud cells grafted adjacent to the embryonic axis without additional notochords behaved similarly to those grafted into the notochord challenge. In

these cases, the morphological distortion of the axial tissues was more severe, placing the host notochord to the extreme contralateral side, while the grafted quail cells were found in distant lateral regions of the operated side or migrated into the limb. Myotomal muscle on the operated side of the embryo was reduced in size compared to the unoperated side of the embryo, but was also populated with thousands of quail cells in both the dorsal and ventral epaxial domains. In 3 of 14 cases, a small number of quail cells were found in the cartilage models, always a small fraction of the total number of quail cells present in all tissues (less than 0.5%). In these cases, sections were not taken for immunocytochemistry, preventing any conclusions about myosin expression.

Response of intact chimeric limb tissue to axial organs

To test whether the inability of dissociated cells to respond to notochord signals may result from the digestion of receptor molecules from the cell surface, the dissociation step was omitted and grafts of whole tissue were made. Quail cells of the stage 21 chimeric limb bud grafted as an intact mass of tissue are also incapable of producing cartilage when challenged with cartilage inducing signals from the notochord. In cases where the limb tissue was grafted in place of somite I and II either in the presence of ectopic notochord (n=4) or in its absence (n=6), no quail cells were observed to contribute to the large ectopic formations of cartilage composed exclusively of chick cells. Instead, many thousands of quail cells are found exclusively in differentiated skeletal muscle tissue in both the dorsal and ventral epaxial domains. This tissue is also oriented parallel to the embryonic body axis. While the quail cells often form separate domains of skeletal muscle tissue, they can also join the formation of chimeric muscle tissue populated by additional chick cells.

Differentiation of dissociated limb cartilage cells in the axial domain

To determine if cells capable of forming cartilage in the limb regions are able to do so in the axial environment, whole quail limbs were dissociated and grafted adjacent to the axis. Figure 4.8 Panel A, shows a representative example. In only 1 of 6 cases were quail cells detected in a recognizable cartilage structure. Panels C and D show that the quail cells were formed into a cartilage tissue that was independent of the host cartilage model, and composed exclusively of quail cells. In the remaining 6 of 7 cases, quail cells populated large skeletal muscle tissues in both the dorsal and ventral epaxial domains, but did not differentiate as cartilage. Near the edges of the graft, the quail cells were formed into longitudinally organized chimeric muscles. In panel B, the subectodermal dermis on the operated side of the embryo contains quail cells and is substantially thicker than the contralateral control side. It is formed into structures resembling incipient feather germs of the limb ectoderm, and may have been produced by grafting limb dermal cells to an axial position. This extra thickening of the dermis was always seen in conjunction with a depletion of the loose mesenchymal cells that are present between the dermis and the underlying myotome. It is possible that the grafted limb muscle cells in the axial region influence the rate of proliferation or the morphological characteristics of the host axial dermal cells underlying the axial ectoderm.

Response of cytochalasin D treated chimeric limb cells to ectopic notochord grafts

Quail cells that were grafted as dissociated suspensions of chimeric limb cells and then challenged with overlying notochords, were often displaced to the extreme lateral margin of the operated side of the chimera, and could often be found in the limb musculature. It is possible that the inability of these cells to join in the formation of axial cartilage tissue is a result of their migration away from the notochord implant. To temporarily disable the cytoskeletal apparatus in the quail muscle precursor cells, the

dissociated suspension of cells was incubated in the presence of cytochalasin D. In 5 of the 5 chimeras constructed in this way, no quail cells were seen to enter the cartilage model. Figure 4.9 shows the results of one such experiment. Panels A and B show the large mass of quail muscle formed in the ventral epaxial domain. Boxed areas C and D are magnified in Panels C and D below, showing quail nucleoli in myotubes (C) that strongly express myosin heavy chain (D). Well formed muscle tissue was present in both the dorsal and ventral epaxial domains. Vehicle (DMSO) treated controls also produced no quail cartilage cells.

Discussion

Expression of transcription factors and the acquisition of myogenic memory

The performance of marked limb muscle precursors in the notochord challenge assay reveals a population of determined muscle precursor cells present in the limb at early stages of development. These cells express the Pax3 mRNA while enroute to their destination in the limb field (Bober, et al., 1994b; Goulding, et al., 1994; Williams and Ordahl, 1994). Overt expression of myoD and myf5 is not seen until two days after the presence of these determined cells in the limb can be demonstrated (Williams and Ordahl, 1994). Thus, determined skeletal muscle cell precursors employ a mechanism for the establishment and maintenance of "myogenic memory" that is antecedent to, and independent of, the overt expression of members of the myogenic bHLH family (Figure 4.10). Previous experiments in which early mouse limb buds were grown as explants in vitro also identified a set of muscle precursor cells present in the limb prior to myogenic factor expression (Sassoon, et al., 1989; Tajbakhsh and Buckingham, 1994).

It would appear then, that a later role in determination is reserved for the myogenic factors, possibly in stabilizing myogenic memory through cross- and auto-activation

mechanisms (Braun, et al., 1989a; Thayer, et al., 1989; Edmondson, et al., 1992). The MRFs are incapable of stabilizing myogenic memory in the embryo until earlier steps of specification have occurred. Expression of a mouse myoD cDNA under the control of the B-actin promoter in transgenic mice does not convert any of the tissues of the embryo to differentiated skeletal muscle, although ectopic activation of myogenin expression does occur. Although this misexpression results in death of the embryo at mid-gestation, formation of the primary germ layers and other organ primordia proceeds normally (Faerman, et al., 1993). This suggests that earlier steps in specification (such as the expression of Pax3 in the paraxial mesoderm, or the inactivation of myogenic repression) are a pre-requisite for myogenic differentiation driven by the myogenic bHLH factors.

Expression of the Pax3 mRNA is not sufficient for the establishment of myogenic memory in all cells of the paraxial mesoderm. Its action is closely dependent on the morphological state of the cell in which it is expressed. In the tightly packed mesenchyme of the segmental plate and the epithelium of the immature somite, Pax3 expression may maintain cells in an ambiguous state that precedes muscle determination but is susceptible to cartilage induction by the notochord. Only in actively migrating mesenchymal cells is Pax3 expression correlated to an irreversible myogenic memory that is insensitive to cartilage inducing signals from the notochord.

Morphogenesis of the cartilage model subverts a small population of unstably committed cells

Previous experiments with chimeric limb buds showed that after dissociation and repackaging into an ectodermal jacket, a small number of quail muscle precursors could be drawn into the cartilage model. While only a small fraction of the total number of quail cells were able to do this, the finding was consistent in all 12 experiments in the original paper (Kieny, et al., 1981). Experiments placing undissociated fragments of tissue from

the dorsal and ventral pre-muscle domains into the chondrogenic core of the limb bud in a separate chick host also showed some presence of quail cells in the cartilage model, but entrance into the cartilage model was not as consistent (Wachtler et al., 1981). We have extended these conclusions by returning somite derived cells to their axial domain, placing them in a more rigorous cartilage signalling context that the majority of cells respond to when present in the somite. The few instances of quail limb muscle cells entering the cartilage model in the experiments reported here (4 of a total of 33 experiments) are consistent with the conclusions of both previous authors that while the vast majority of somite derived cells in the limb are determined to form limb muscle, a small population of cells retains the ability to respond to notochord cartilage signalling. The loss of response to notochord signals can thus be judged to be an aspect of muscle cell determination.

In the limb field, signals for the morphogenesis of cartilage models may be reconstituted in the limb mesenchyme repacking experiments. Pre-muscle cells in the limb may therefore come under the influence of these signals after spatial displacement. The few limb muscle precursor cells that were recruited to axial cartilage in the notochord assay could not do so autonomously; it appears that they must join the morphogenetic organization of host cartilage cells migrating from the adjacent sclerotomes prior to cartilage differentiation. Although they are morphologically indistinguishable from the surrounding host cartilage, in some instances expression of myosin heavy chain can be detected. Pre-cartilage cells from the limb do not respond to axial morphogenetic signals; limb and axial cartilage elements also have different requirements for a functional Pax1 gene product (Dietrich and Gruss, 1995). These results imply that the morphogenetic programs for axial and limb cartilage employ a cell-intrinsic sorting mechanism that is able to discriminate between axial and limb cartilage, but does not recognize cells specified for the muscle phenotype as foreign. Morphogenetic organization of pre-cartilage cells may be a pre-requisite for differentiation.

Emerging determined muscle precursors express myosin heavy chain in the cartilage model

Limb muscle precursors originate in the lateral half of the somite (Ordahl and Le Douarin, 1992). Expression of the Pax3 mRNA is specifically up-regulated in this compartment of the somite as the somite matures, and high level expression is maintained as precursor cells leave the somite and enter the limb field (Bober, et al., 1994b; Goulding, et al., 1994; Williams and Ordahl, 1994). Muscle precursors from the lateral half of stage II somites exhibit two behaviors in the notochord challenge assay. Small, nerve dependent myoclusters similar to those seen from dorso-medial grafts of stage II somites were produced from 60% of the grafts in this assay. The numbers of cells in each instance slightly exceeded that produced from the dorso-medial somite. A second population of muscle cells unique to the lateral somite was unresponsive to its position in a cartilage model and expressed myosin heavy chain while deeply embedded in this tissue. This second type of muscle cell is apparently independent of any neural influence, as it can differentiate as skeletal muscle in a region that is distant from the known muscle promoting influences of the dorsal neural tube and overlying ectoderm. Although morphologically indistinguishable from true cartilage cells, they continue to express myosin heavy chain. These cells are limited in mitotic potential and can express myosin heavy chain either weakly or strongly .

A role for the notochord or notochord factors in muscle specification has recently been argued (Buffinger and Stockdale, 1994; Johnson, et al., 1994; Munsterberg and Lassar, 1995; Stern and Hauschka, 1995; Currie and Ingham, 1996; Hammerschmidt, et al., 1996; Pownall, et al., 1996; Weinberg, et al., 1996). *Sonic hedgehog* is thought to prepare the paraxial mesoderm for the muscle inducing properties of the dorsal neural tube (Munsterberg). Targetted disruption of the *sonic hedgehog* gene causes gross morphological defects, but the onset of myogenic factor expression appears to be normal

(Chiang, et al., 1996). Thus the notochord may provide trophic factors for muscle cells or help maintain the dorsal neural tube in an actively signalling state, but the initial steps of muscle specification in the paraxial mesoderm proceed normally in its absence. Since greater than 99% of stage II lateral somite cells alter their fate to cartilage, inductive signalling from the notochord appears to specify cartilage, not muscle.

Myogenic determination is cell intrinsic

Placement of cells from the primitive streak, areas adjacent to Hensen's node, or the unsegmented paraxial mesoderm into the limb bud identified the limb as a permissive environment for competent cells to differentiate as skeletal muscle. Cells in such early stages of specification may very well find interactions with the limb mesenchyme or overlying limb ectoderm to induce or maintain their commitment to the skeletal muscle fate.

Limb bud ectoderm is known to effect the development and patterning of tissues within the limb (MacCabe et al., 1974; Riddle et al., 1995; Yang and Niswander, 1995), and limits the growth of the chondrogenic region, allowing muscle cell precursors to migrate into position (Solursh and Reiter, 1988). Chimeric limb buds were stripped of ectoderm to remove any influence from this component of the limb bud. The dorsal and ventral regions of the remaining mesenchymal component were separated from the central chondrogenic region and then dissociated with trypsin to disrupt any specific cell/cell contacts between the somite-derived muscle precursors and the limb mesenchyme. Dissociation to near uniform monocellularity was confirmed by inspection under the microscope.

It is a formal possibility that specific cell/cell associations with cells of the limb mesenchyme may have been reconstituted during the reaggregation process. It is also possible that cells of the limb mesenchyme secrete soluble factors that instruct the somite derived cells to differentiate as skeletal muscle. To eliminate both of these possibilities,

future experiments will label the cells of the brachial paraxial mesoderm with fluorescent vital dyes (CFSE), and allow their migration into the limb bud. The chimeric limbs will then be dissociated, and subject to fluorescence activated cell sorting, prior to reaggregation and grafting into the notochord challenge environment.

Trypsin digestion is routinely used to strip the cell surface of receptor proteins, and the inability to respond to cartilage signals may have resulted from this harsh treatment. However, both dissociated chimeric limb cells and undigested chimeric limb tissue grafted into the notochord challenge produced very few quail cartilage cells. These results combined suggest that the cells have withdrawn any signal-sensing apparatus required to respond to cartilage inducing signals, and point to a cell intrinsic mechanism for the maintenance of myogenic memory in migrating limb muscle precursor cells.

Myogenic memory may be maintained in stages of specification prior to myogenic bHLH expression by withdrawal of receptors on the cell surface for cartilage signalling molecules, and may be subverted by hyperactive cartilage signalling. For example, local expression of BMP4, or a BMP2 supplemented culture medium, can suppress the formation of muscle derived from the lateral somite (Duprez et al., 1996; Pourquie et al., 1996). Forced expression of the BMP receptor in muscle precursor cells of the limb (Niswander, pers. comm.), release of BMP from sequestration by ablation of the *noggin* gene (Zimmerman et al., 1996) and Harland, pers. comm), and removal of the limb ectoderm (Solursh and Reiter, 1988) all result in the expansion of limb chondrocytes and a diminution of limb skeletal muscle. Progression of the cartilage/bone differentiation program can even reach a point where it is dominant over forced expression of myogenic bHLH factors (Filvaroff and Derynck, 1996). The vertebrate homologs of *patched* and *smoothened* (Goodrich et al., 1996; Stone et al., 1996), receptors for the sclerotome inducing signal *sonic hedgehog* (Fan, et al., 1995), are expressed in the ventral somite region, and excluded from the dorsal somite epithelium which forms skeletal muscle.

Thus, early stages of skeletal muscle specification may require insulation from the effects of surrounding cartilage inducing influences by down-regulation of receptor expression. It is interesting to note here that forced expression of Pax3 in cells of the lateral plate and paraxial mesoderm can rescue BMP-mediated suppression of muscle differentiation (Maroto and Lassar, pers. comm.).

Determination of muscle precursor cells is not caused by motility

Quail limb muscle precursor cells taken from late stages of limb bud development migrate back into the limb when grafted into the axial domain (Mauger and Kieny, 1980). It is possible that the migration of limb muscle precursor cells places them in regions of the embryo that are permissive for the differentiation of skeletal muscle, or specify their fate as skeletal muscle. Migration may also place somite cells outside the cartilage inducing effects of the notochord. This mechanism would require the presence of a chemo-repulsive agent that limb muscle precursor cells are able to sense. The floor plate of the neural tube is known to express netrin, (Serafini et al., 1994), a chemo-repulsive agent for commissural neurons in the spinal cord (Colamarino and Tessier-Lavigne, 1995). Netrin is also expressed by the dermomyotome (Kennedy et al., 1994). Pax3 expression is maintained in older embryos at low levels in the limb when axial expression is strongly suppressed (unpublished observations), suggesting continued out-migration of Pax3 expressing cells from the body axis to the limb bud.

Migration of muscle precursors is strongly affected by expression of the Pax3 gene. Homozygous mutations of Pax3 prevent the migration of limb muscle precursor cells (Franz, et al., 1993; Bober, et al., 1994b; Goulding, et al., 1994), and delay migration of neural crest cells from the neural tube (Moase and Trasler, 1990). Up-regulated expression of Pax3 in the lateral half of the somite may be responsible for the expression of c-met in the lateral half of the somite (Bladt, et al., 1995), as binding sites for Pax3 in the c-met

promoter have recently been identified (Epstein, et al., 1996). c-met may mediate the epithelial to mesenchymal transition of lateral half somite cells through the action of HGF/scatter factor, which is expressed in the nascent limb bud (Bladt, et al., 1995). Mutations in c-met also prevent the emigration of somite cells into the limb (Bladt, et al., 1995), and forced expression of c-met in somites of the inter-limb regions can cause the epithelial to mesenchymal transition normally seen only in limb level somites (Brand-Saberi et al., 1996). Pax3 expression may also act to prevent the differentiation of muscle precursor cells during migration. Forced expression of Pax3 in C2C12 myoblasts inhibits muscle differentiation by down-regulating myogenin expression while myoD and myf5 expression are unaffected (Epstein et al., 1995).

Temporarily disabling the cytoskeletal motility apparatus of limb bud muscle precursor cells by incubation with cytochalasin D was unable to prevent the exclusive differentiation of quail cells as skeletal muscle. Thus, mobility of muscle precursor cells does not appear to be required for determination to make skeletal muscle. It is possible that the exposure was too brief, and that notochord signals could not affect the cells irreversibly before they had metabolized cytochalasin D. Vertebral cartilage differentiation does not occur until approximately day 7 in the chick embryo, so there was plenty of time for this to occur. In spite of these caveats, it appears that cellular motility is not the basis for skeletal muscle determination in limb bud muscle precursors.

A possible role for Pax genes in skeletal muscle specification

Retroviral infection has been used to test the effects of ectopic expression of Pax3 in different regions of the developing embryo. Cells of the neural tube and lateral plate, when infected with the Pax3 retrovirus in explant culture, produce differentiated skeletal muscle, without the influence of any accompanying signalling tissues. Up-regulation of endogenous myf5 and myoD follows closely behind as an early response to this infection.

(Maroto and Lassar, pers. communication). Brain and neural tube cells appear to have a "myogenic tendency" as evidenced by overt myf5 expression in regions near developing motor neurons in the neural tube and and in the mesencephalon and later in the posterior hypothalamus (Tajbakhsh et al., 1994; Tajbakhsh and Buckingham, 1995). Misexpression of Pax3 in the muscle promoting environment of the neural tube (Munsterberg and Lassar, 1995) may enhance this tendency. However, the formation of skeletal muscle from the lateral plate, a tissue that has never convincingly demonstrated myogenic potential, is strong evidence that Pax3 may be able to cause the specification of muscle cell type in mesoderm. MRF expression in response to Pax3 expression, confirms that the MRFs function late in the specification pathway of skeletal muscle, downstream of the effects of Pax3.

The formation of myotomal muscle is aberrant in Splotch mice, while limb muscle is non-existent (Franz, et al., 1993). When lateral half somites from Splotch mice are manually placed into the chick limb bud environment, they are capable of differentiation as skeletal muscle (Daston, et al., 1996). Cells from Splotch somite grafts that did not differentiate as skeletal muscle were not analysed in detail in this report, and the mutant cells were not tested for ability to respond to cartilage inducing signals. Curiously, grafts of the lateral half of the segmental plate from Splotch mice are unable to differentiate as muscle in the limb bud environment, unlike wild-type grafts of quail segmental plate (Wachtler, et al., 1982), or in vitro cultures of lateral half segmental plates (Gamel, et al., 1995). These results demonstrate that differentiation as skeletal muscle can occur in the absence of a functional Pax3 gene product, but that the full complement of muscle differentiation competence is not found in the segmental plate when Pax3 is mutant.

If Pax3 functions in the specification of skeletal muscle in the embryo, why are myotomal muscles still formed in the Splotch mouse? Another member of the paired-domain family of transcription factors, Pax7, which is closely related to Pax3, is expressed

in the somite dermomyotome, but is not expressed in the migrating muscle precursors. Pax7 is the only other member of the Pax family of transcription factors that has an octapeptide repeat flanked by a paired domain DNA binding motif and a complete paired-type homeodomain (Strachan and Read, 1994). Expression of the Pax7 gene product in the dermomyotome of Splotch mice may be able to compensate for the loss of Pax3 expression. Pax7 has recently been disrupted in the mouse embryo, producing a craniofacial defect, but no apparent skeletal muscle deficit (Mansouri et al., 1996). Crossing the Pax7 mutation into the Splotch background will illuminate the role of paired domain transcription factors in the specification of skeletal muscle.

Acknowledgements

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Figure 4.1. Preparation of the chimeric limb bud, as seen through the stereomicroscope.

Panel A shows a dorsal view of the stage 12HH host chick embryo at the level of the brachial segmental plate. Black ink has been injected underneath the embryo to improve visual contrast for surgery. The ectoderm has been cut between the neural tube and the segmental plate, and is reflected with a small scalpel blade after brief digestion with pancreatin.

Panel B shows the area after the segmental plate has been removed by aspiration with a micro-pipette.

Panel C is a ventral view of the stage 12HH donor quail embryo pinned out in a dissection dish. The segmental plate has been removed and carbon particles attached to the rostral tip of the plate as an orientation marker.

Panel D shows the donor fragment grafted into the excavated area of the host segmental plate. The orientation marker can be seen at the rostral tip of the grafted donor tissue.

Panel E shows the stage 22HH chimeric embryo after 2 days of incubation. The ectoderm of the chimeric limb has been stained with Nile blue sulfate.

Panel F is a close up view of the stained chimeric limb bud, showing that the morphology of structures such as the ectodermal ridge is normal.

Figure 4.1

Figure 4.2. Preparation and implantation of a segmental plate "organoid" from dissociated chimeric limb bud cells, as seen through the stereomicroscope.

Panel A shows the ectoderm of the chimeric limb stained with Nile blue; there is a midline incision in the dorsal ectoderm, that will aid in digesting and peeling away the ectodermal jacket from the underlying mesenchymal tissue.

Panel B shows the dorsal and ventral mesenchymal regions containing the quail cells being sliced away from the centrally placed chondrogenic cord of the limb bud, which is comprised primarily of quail cells.

Panel C shows one of the removed mesenchymal regions removed to a culture dish, where it will be dissociated by incubation with trypsin for 30 minutes at 37C.

Panel D shows the cells from the future muscle regions of the limb dissociated in suspension after trituration with a micropipette.

Panel E shows the dissociated cells packed into the tip of a glass needle after centrifugation at 3000 rpm for 5 minutes.

Panel F shows the cells of the chimeric limb bud extruded into a culture dish, forming an "organoid" that is of roughly the same dimensions as the segmental plate that it will replace in a new chick host embryo.

Panel G shows a new stage 12HH chick host embryo that has had the segmental plate removed to receive a graft of the limb cells.

Panel H shows the "organoid" comprised of limb mesenchyme cells extruded into the cavity left after removal of the host segmental plate.

Panel I shows the host ectoderm in place over the grafted limb cells.

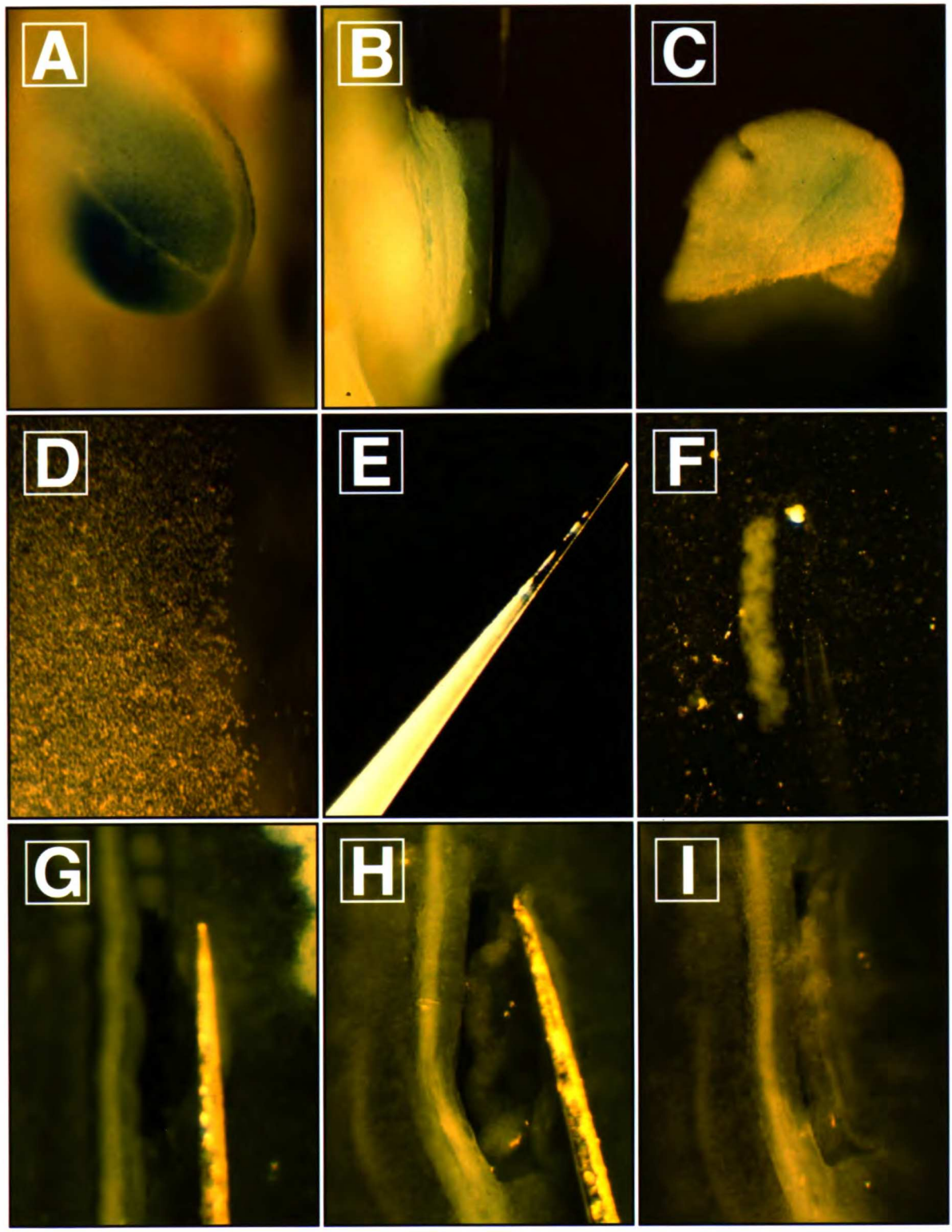


Figure 4.2

Figure 4.3. The vast majority of dorso-lateral stage II somite cells form cartilage in response to notochord signals.

Panel A (27X) shows a Feulgen stained cross-section of an 8 day chimeric embryo which received a graft of the dorso-lateral cells from the stage II somite overlaid with two additional notochords. The three surrounding notochords are marked with asterisks. Note the presence of a large domain of ectopic cartilage tissue, and the distortion of the normal morphology of the tissues due to the ectopic notochord positions. The area shown in panel C is boxed.

Panel B shows the MF20 stained section adjacent to Panel A, also at 27X magnification. Large regions of myosin-positive skeletal muscle are stained with the antibody and labelled "host muscle".

Panel C shows the boxed region in Panel A at 560X magnification. The large nucleolar marker is pointed out with arrows; these quail cells are located in the cartilage model.

Panel D shows the boxed region in Panel B at 560X magnification; the regions containing quail cells are in an area of the cartilage model that does not react with the MF20 antibody.

Figure 4.4. Dorso-lateral cells from the stage II brachial somite also form small clusters of nerve-dependent myocytes in response to notochord signals.

Panel A (27X) shows a Feulgen stained cross-section of an 8 day chimeric embryo which received a graft of dorso-lateral cells from the stage II somite, overlaid with two additional notochords. The ectopic cartilage surrounding the supernumerary notochords contributes to the overall distortion of the tissue morphology seen here.

Panel B (27X) shows an adjacent MF20 stained cross-section with host muscle masses marked. The large arrow indicates a small myocluster produced by grafted quail cells. This boxed area is magnified in Panel D.

Panel C shows the boxed area in Panel A at 560X magnification. A small cluster of quail cells (arrows) is present in an area near the future intervertbral foramen, the conduit for the spinal nerve.

In Panel D, the boxed area in Panel B is magnified at 560X. Here, the regions shown to be populated by quail cells in Panel C are myosin heavy chain positive, indicating that a small "myocluster" has formed from the grafted cells.

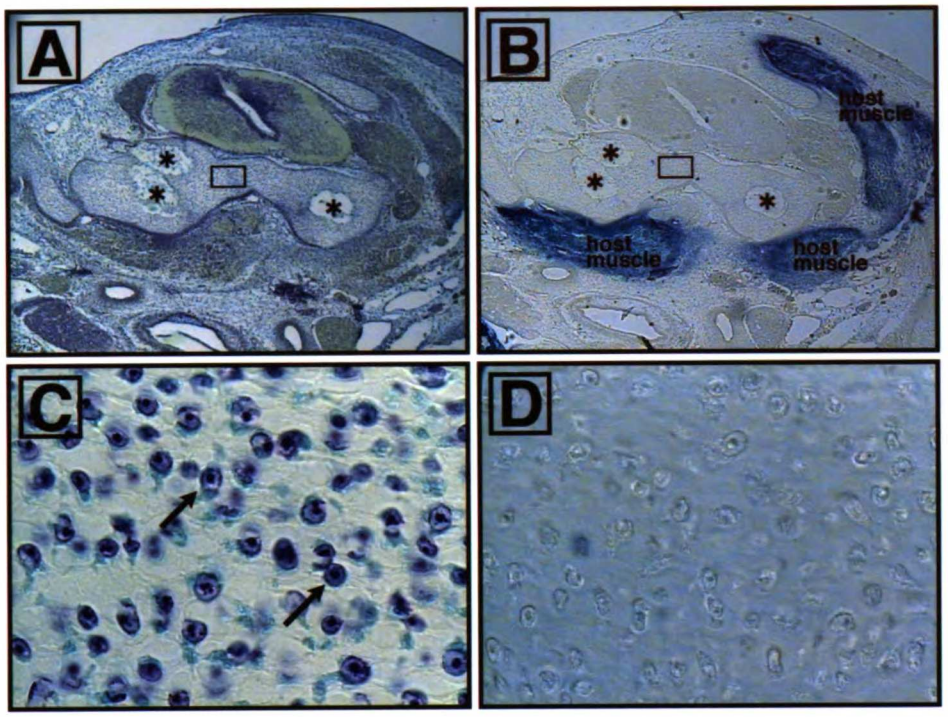


Figure 4.3

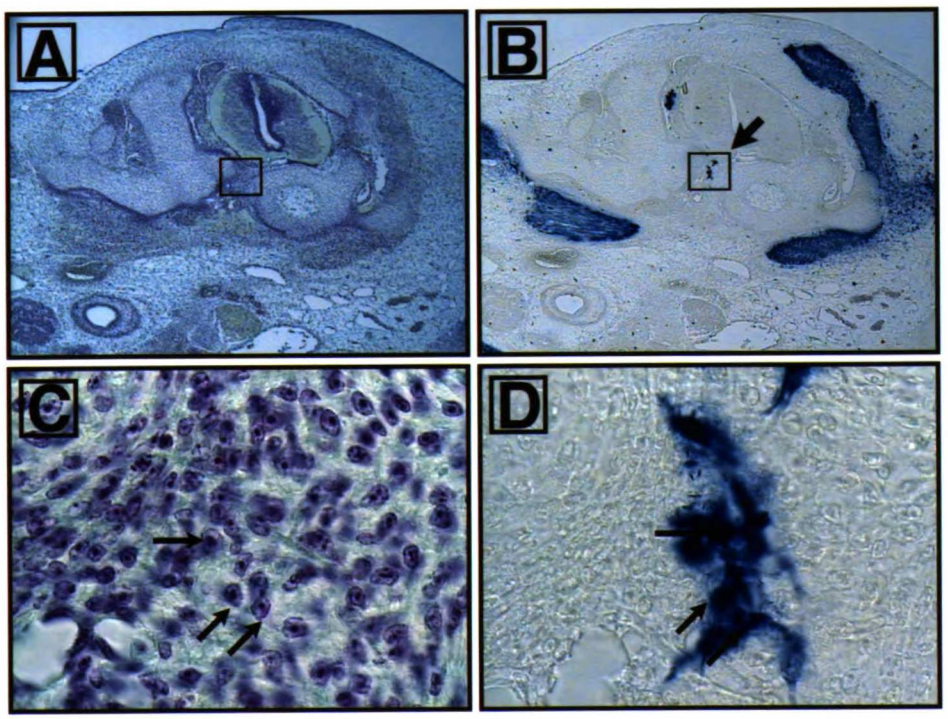


Figure 4.4

Table 4.1 Dorso-lateral stage II somite cells produce large numbers of cartilage cells and two types of skeletal muscle cells in the notochord challenge.

The five experiments reported here were performed by grafting the dorso-lateral quadrant of brachial stage II somites from quail embryos into a chick host. All of the specimens showed evidence of thousands of quail cells in chimeric vertebral cartilage models. 3 of the 5 examples produced small myoclusters in the ventral epaxial domain, that closely resembled myoclusters from dorso-medial somite grafts reported in Chapter 3. In one case reported here (and in another that was formally excluded due to the escape of graft cells into the dorsal epaxial domain), quail cells present in the cartilage model expressed myosin heavy chain, as detected by the MF20 antibody.

Table 4.1

Dorso-lateral somite challenge
Stage II somite

Specimen	Ventral muscle	# cells in myoclusters	cartilage	# muscle cells in cartilage
DLQ 65	-	0	++	0
DLQ66	-	0	++	0
DLQ68	+/-	61(neural tube)	++	0
DLQ69	-/+	27 (spinal nerve)	++	0
DLQ71	+/-	25 (neural tube)	++	62

Four additional grafts were made with the stage II DLQ. These all contained cells that migrated into the dorsal epaxial domain, and were not included in the analysis (see chapter 3). Of these four, one contained no identifiable muscle, three contained many quail cells that differentiated as muscle in the dorsal epaxial domain and one of these three contained an additional small colony of muscle cells (83) differentiating deep within the cartilage model.

Figure 4.5.1 and 4.5.2 Dorso-lateral grafts from the stage II brachial somite contain a population of myogenic cells that differentiate as muscle deep within the cartilage model.

Panels A (27X) show Feulgen stained cross-sections of two 8 day old chimeric embryos which received grafts of the dorso-lateral stage II brachial somite overlaid with two additional notochords. The ectopic cartilage formed by the notochords is populated by quail cells derived from the graft. The surrounding notochords are marked with asterisks.

Panels B (27X) show adjacent sections stained with MF20. The host muscles are labelled. Within the boxed region, a small population of MF20-positive cells can be seen within the cartilage model.

Panels C show the boxed regions magnified to 560X. Quail cells are clearly contained within the cartilage model.

Panels D show the boxed regions from Panels B magnified to 560X. The MF20 positive cells located entirely within the cartilage model in 4.5.1D are strong expressors of myosin heavy chain, and have the appearance of mononucleate myocytes in the cartilage model. The small cluster of cells in 4.5.2.D are weak expressors of myosin heavy chain, and are morphologically indistinguishable from surrounding differentiated cartilage cells in the cartilage model.

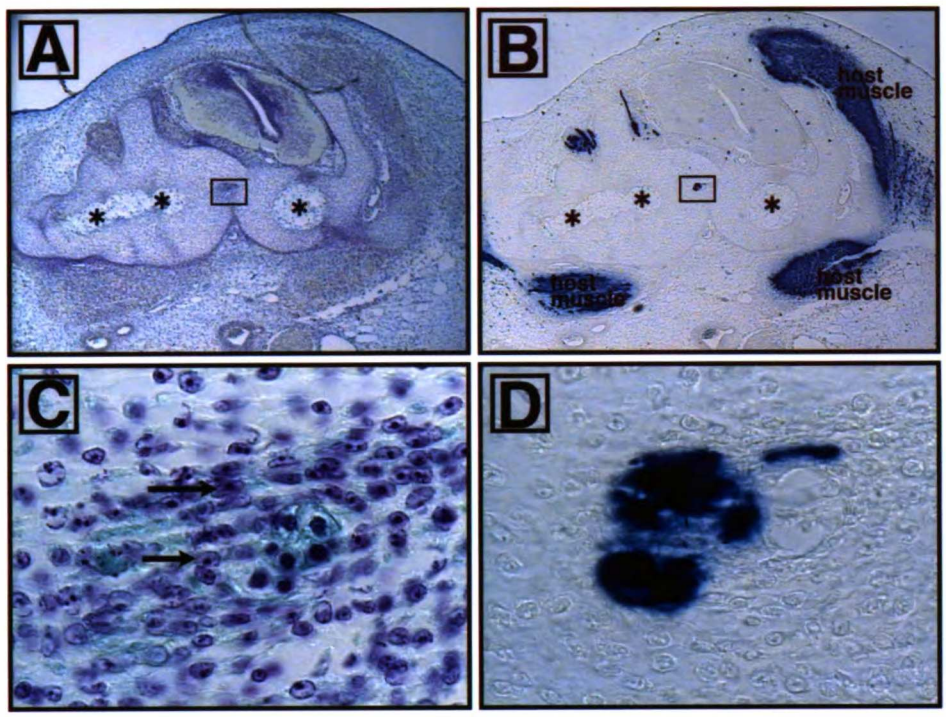


Figure 4.5.1

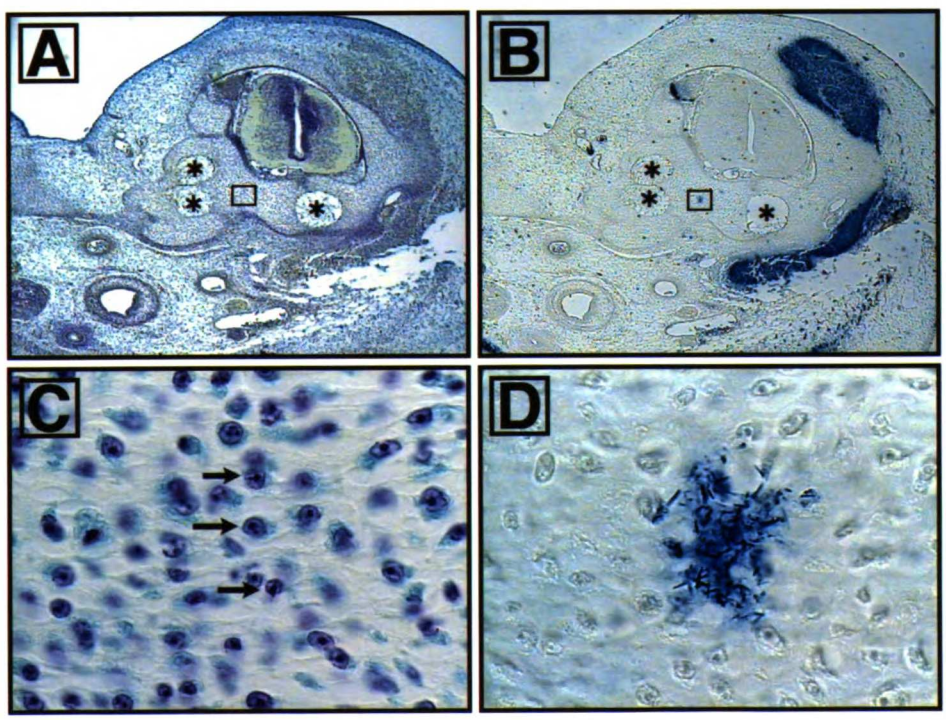


Figure 4.5.2

Table 4.2 Muscle precursor cells in the stage 20-22HH chimeric limb bud are determined to form skeletal muscle in the notochord challenge.

Marked limb muscle precursor cells grafted as an intact fragment of tissue produced only differentiated skeletal muscle, whether challenged with supernumerary notochords or not. Dissociated cells from chimeric limbs produced almost exclusively skeletal muscle, but did give rise to a low incidence of cells in the vertebral cartilage model, usually at or below approximately 0.5% of the total cells grafted into the challenge.
Chimeric limb cells immobilized by treatment with cytochalasin D prior to grafting also produced only skeletal muscle.
Cells from whole quail limbs containing the population of chondrogenic cells from the central region of the limb, have a limited ability to produce cartilage in the axial domain. When they do differentiate as cartilage, they do not form chimeric models, and can only differentiate as an autonomous cartilage element.

Table 4.2

Chimeric limb bud challenge
Stages 20-22HH

Experiment	N	Incidence of cells in cartilage	% of cells in cartilage
Whole limb tissue, no extra notochords	6	0	0
Whole limb tissue, one extra notochord	4	0	0
Dissociated cells, no extra notochords	11	3	.08%, .07%, .16%
Dissociated cells, one or two extra notochords	9	1	0.5%
Dissociated cells, cytochalasin D treated, two extra notochords	3	0	0
Whole quail limb dissociated no extra notochords	5	1	large independent mass
Whole quail limb dissociated one extra notochord	1	0	0

Figure 4.6. Stage 21HH limb muscle precursor cells differentiate as skeletal muscle when challenged with notochord signals.

Panel A (27X) shows a Feulgen stained cross-section of an 8 day old chimeric embryo prepared by replacing the host embryo brachial segmental plate with an "organoid" prepared from dissociated cells from a stage 21HH chimeric limb. The limb cells have been challenged by grafting two additional notochords over them, marked with asterisks. The supernumerary notochords are not surrounded by as much ectopic cartilage as in embryos where only the medial half of a single somite has been removed.

Panel B (27X) shows an adjacent cross-section stained with the MF20 antibody to visualize the expression of skeletal muscle myosin. The large muscle tissues produced from the grafted quail limb cells are labelled with large arrows.

Panel C shows the boxed area in Panel A magnified at 560X. The prominent quail nucleoli are marked with arrows.

Panel D shows the adjacent section magnified to 560X. Here the region populated by quail cells in Panel C strongly expresses myosin heavy chain, and is incorporated into a well defined muscle tissue.

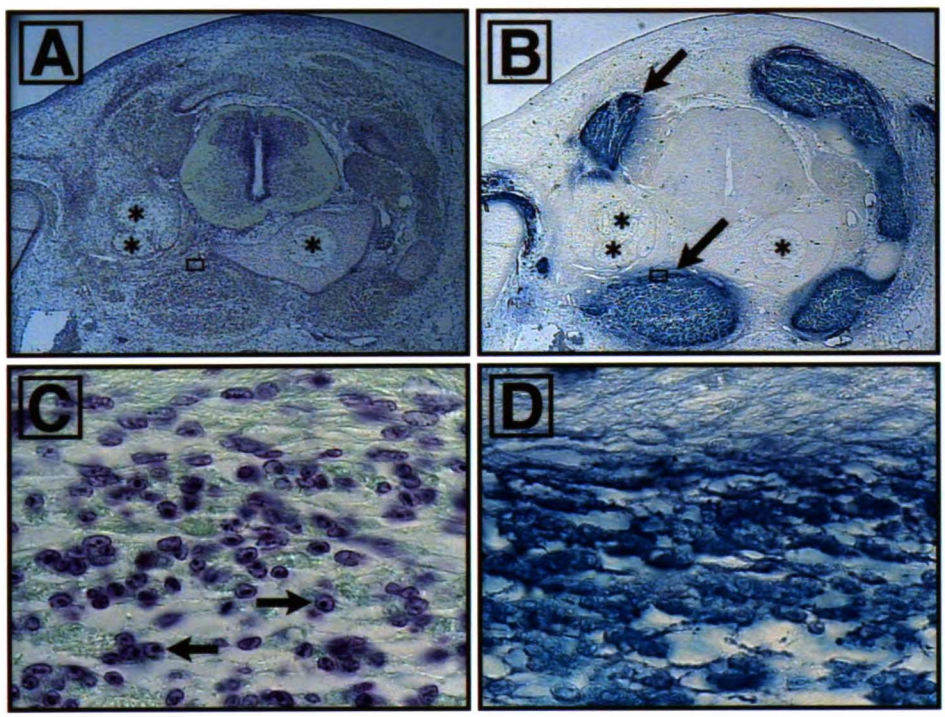


Figure 4.6

Figure 4.7. A small population of stage 21HH muscle precursor cells differentiates as vertebral cartilage in the notochord challenge. Some grafted cells in the cartilage model express myosin heavy chain.

Panel A (27X) shows a Feulgen-stained cross-section from an 8 day old chimeric embryo with graft of dissociated and reaggregated limb cells from a stage 22HH chimeric limb bud challenged with supernumerary and host notochords (marked with asterisks).

Panel B (27X) shows the adjacent section stained with the MF20 antibody to visualize skeletal muscle myosin.

Panel C is a magnification to 560X of boxed area C shown in Panel A. In this case, a small cluster of quail cells can be seen within the cartilage model, and are also incorporated into the perichondrial tissue (arrows). The adjacent panel magnified to 560X is stained with MF20 in Panel D. Here the quail cells in the cartilage model are not expressing myosin heavy chain.

In Panel E, a second population of quail cells is located within the cartilage model (boxed area E in Panel A, magnified to 560X).

Panel F shows that these cells are myosin heavy chain positive, by their reaction to the MF20 antibody.

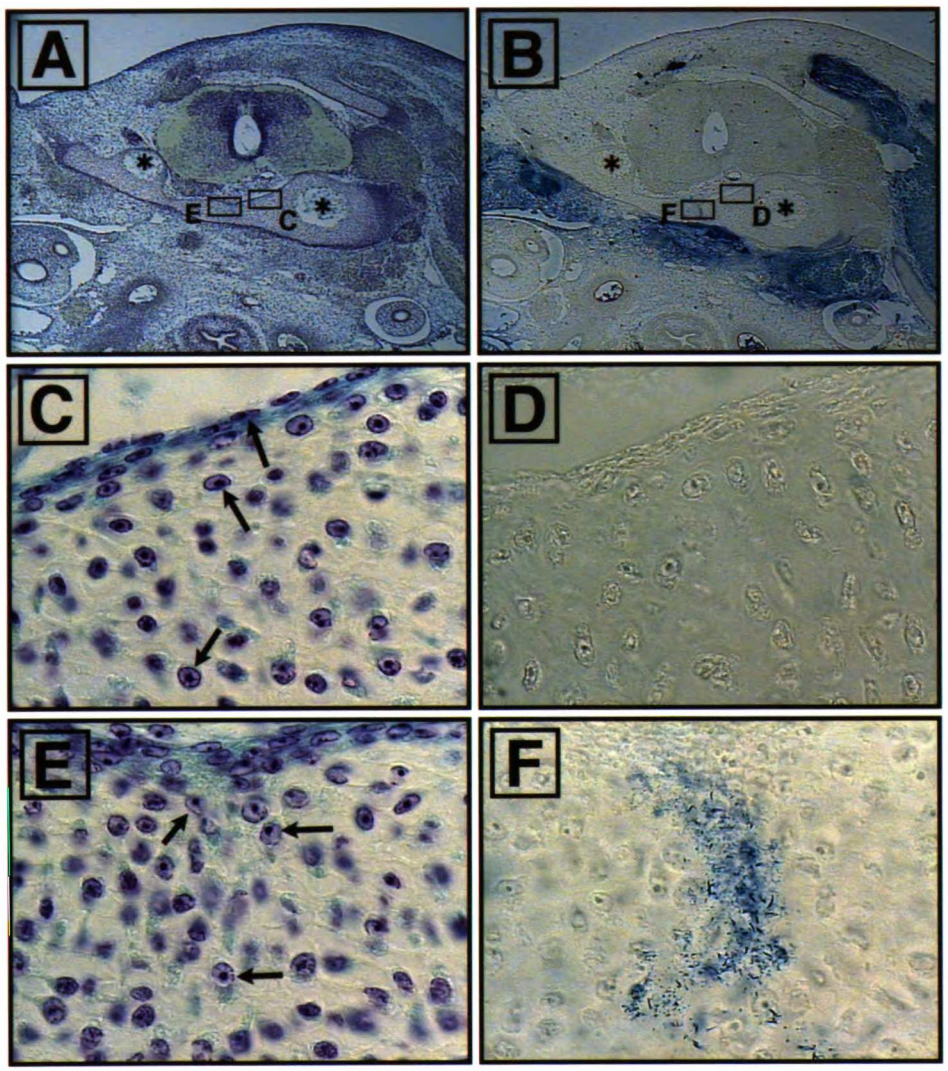


Figure 4.7

Figure 4.8. Limb cartilage cells have a limited ability to differentiate in the axial domain

Panel A (27X) shows a Feulgen stained cross-section of an 8 day old chimera with a graft of dissociated whole quail limb, including cells from the chondrogenic core. Note the absence of the neural arch, pedicle and lamina on the operated side, and the disposition of the ventral neural tube and notochord away from the operated side.

Panel B shows the morphology of the operated region at 56X magnification. Thickening of the sub-ectodermal dermis is seen (arrowhead), along with a depletion of cells in the loose mesenchyme immediately subjacent to it (arrow).

The large, independent cartilage element formed from quail cells is labelled (arrow), and magnified in Panel C (230X). The cartilage cells are surrounded by a continuous perichondrial layer.

Panel D is a magnification at 350X showing the cellular detail of this tissue. Quail cells arrow are seen in both the hypertrophied cartilage in the center of the tissue and in the perichondrium.

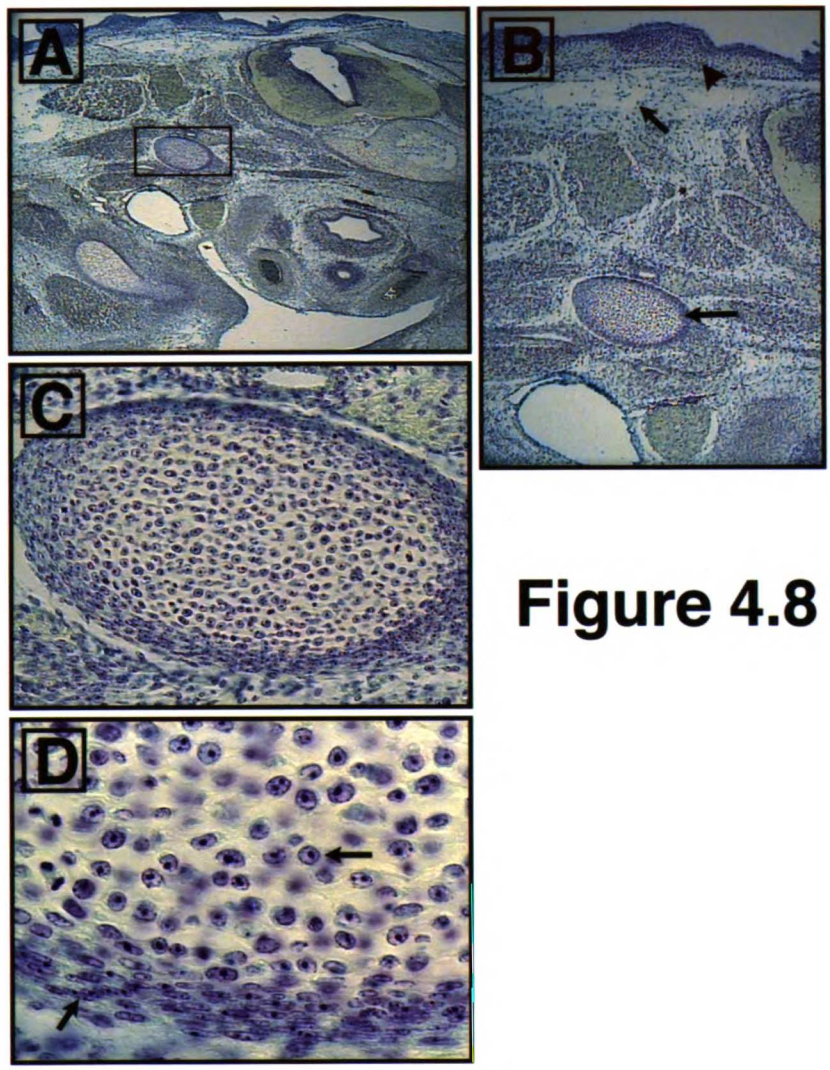


Figure 4.8

Figure 4.9. Cytochalasin D-treated limb muscle precursor cells differentiate as skeletal muscle in the notochord challenge environment.

Panel A (27X) shows a Feulgen stained cross-section of an 8 day old chimeric embryo which received a graft of chimeric limb cells implanted into the notochord challenge environment after a 30 minute treatment with cytochalasin D.

The adjacent section in Panel B (27X) stained with MF20 shows the location of cells that have differentiated as skeletal muscle. A large mass of graft derived muscle cells is indicated by the large arrow.

Panel C shows the boxed area in Panel A magnified to 560X. The quail cells in this panel are clearly arranged into multinucleate myotubes, arranged in a larger structure of organized skeletal muscle tissue.

Panel D (560X) shows that the cells pictured in Panel C are expressing myosin heavy chain, by reaction with the MF20 antibody.

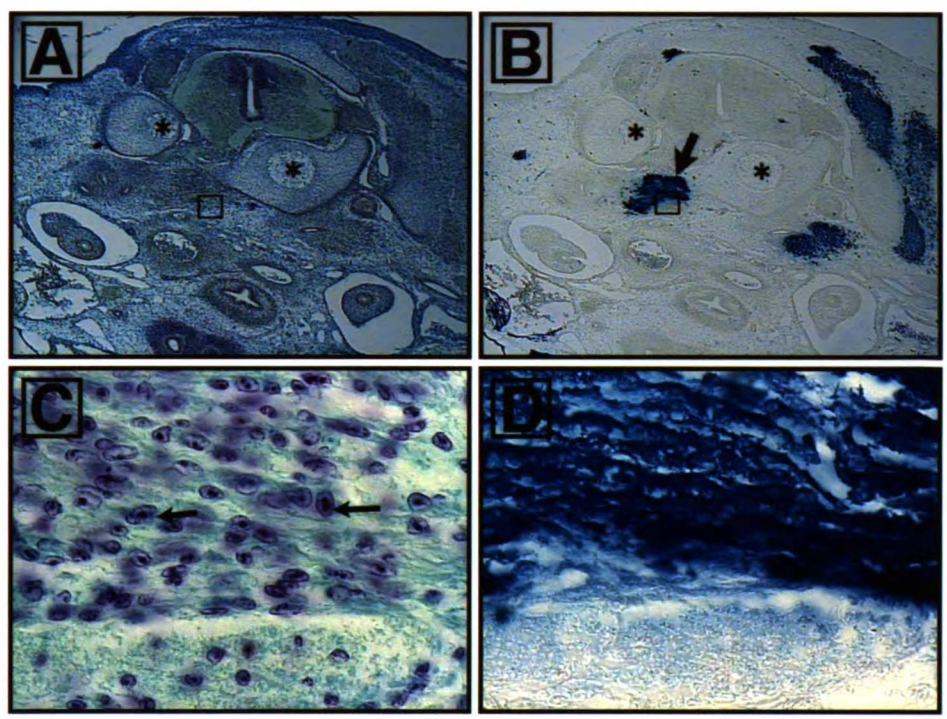


Figure 4.9

Figure 4.10. A schematic representation of progression through the myogenic lineage.

Pax3 expression begins at gastrulation, and is found in cells of the segmental plate and somites, where paraxial mesoderm cells are competent to form skeletal muscle, but can be converted to other cell types by notochord challenge. Cells enter the determined phase of specification while continuing to express Pax3, but have now enacted a motility behavior that is likely regulated by the expression of c-met and the presence of scatter factor/HGF in the nascent limb bud. Expression of the myogenic bHLH transcription factors is a late event in the determination of cells in the embryo, and initiation of myogenic bHLH expression immediately precedes skeletal muscle cell differentiation.

Pax-3 expression marks stages of myogenic specification prior to overt MDF expression

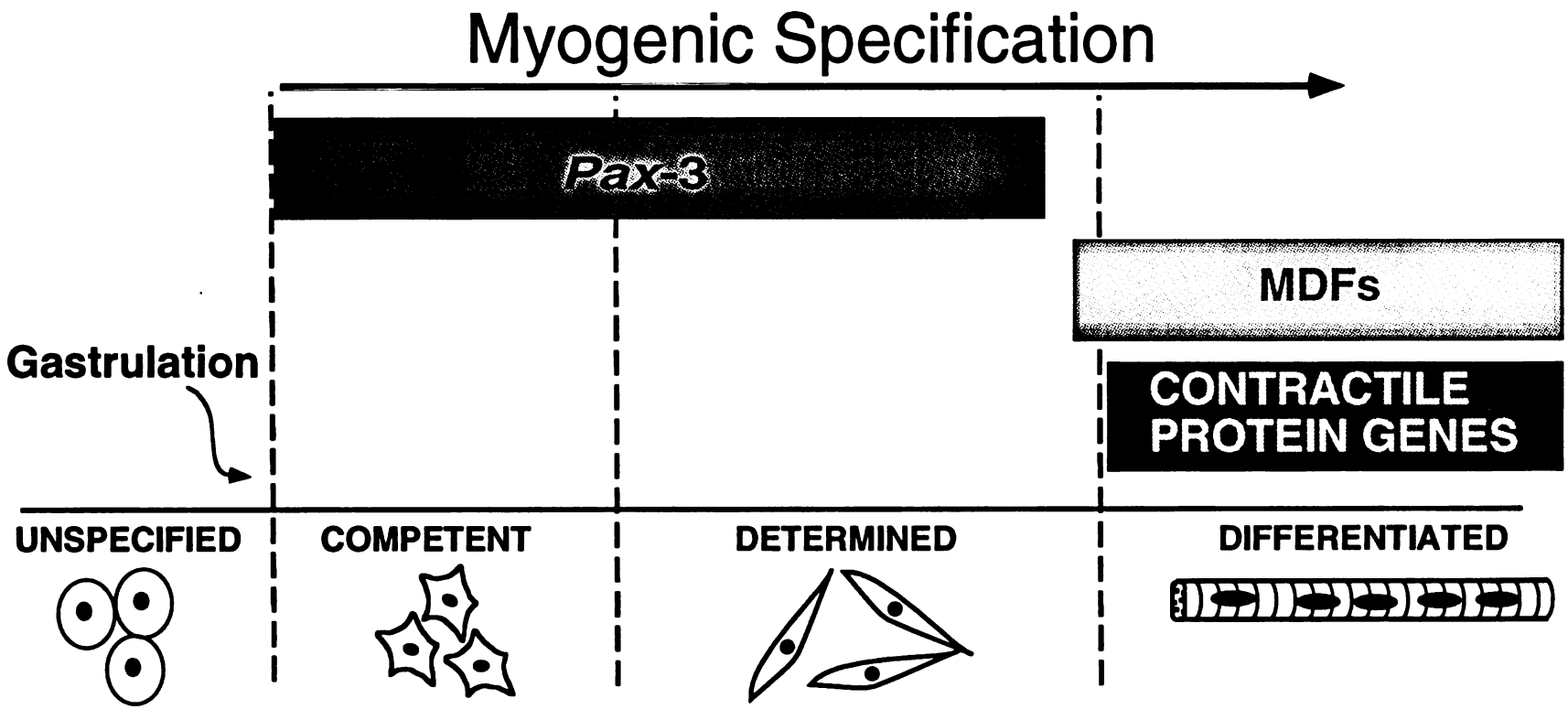


Figure 4.10

BIBLIOGRAPHY

Aoyama, H. (1993). Developmental plasticity of the prospective dermatome and the prospective sclerotome region of the avian somite. *Dev. Growth and Diff.* **35,** 507-519.

Aoyama, H. and Asamoto, K. (1988). Determination of somite cells: independence of cell differentiation and morphogenesis. *Development* **104,** 15-28.

Araki, I., Saiga, H., Makabe, K. W. and Satoh, N. (1994). Expression of AMD1, a gene for a MyoD1-related factor in the ascidian Halocynthia roretzi. *Roux's Arch. Dev. Biol.* **203,** 320-327.

Bandman, E. (1985). Myosin isoenzyme transitions in muscle development, maturation and disease. *Int. Rev. Cytol.* **97,** 97-131.

Bate, M. (1990). The embryonic development of larval muscles in Drosophila. *Development* **110,** 791-804.

Bate, M., Rushton, E. and Currie, D. A. (1991). Cells with persistent twist expression are the embryonic precursors of adult muscles in *Drosophila. Development* **113,** 79-89.

Beddington, R. S. P. and Martin, P. (1989). An in situ transgenic enzyme marker to monitor migration of cells in the mid-gestation mouse embryo: somite contribution to the forelimb. *Mol. Biol. Med.* **6,** 263-274.

Bellairs, R. (1986). The primitive streak. *Anat. Embryol.* **174,** 1-14.

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L. and Weintraub, H. (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61,** 49-59.

Beresford, B. (1983). Brachial muscles in the chick embryo: the fate of individual somites. *J Embryol exp Morph* **77,** 99-116.

Berleth, T., Burri, T., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M. and Nusslein-Volhard, C. (1988). The role of localization of bicoid RNA in organizing the anterior pattern of the *Drosophila* embryo. *Embo J.* **7,** 1749-1756.

Bischoff, R. and Holtzer, H. (1969). Radioautographic study of the relation between mitosis and the subsequent fusion of myogenic cells in vitro. *J. Cell Biol.* **41,** 188-203.

Black, B. L., Martin, J. F. and Olson, E. N. (1995). The mouse MRF4 promoter is trans-activated directly and indirectly by muscle-specific transcription factors. *J. Biol. Chem.* **270,** 2889-2892.

Bladt, F., Rietbmacher, D., Isenmann, S., Aguzzi, A. and Birchmeier, C. (1995). Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* **376,** 768-771.

Bober, E., Brand-Saberi, B., Ebensperger, C., Wilting, J., Balling, R., Paterson, B. M., Arnold, H.-H. and Christ, B. (1994a). Initial steps of myogenesis in somites are independent of influence from axial structures. *Development* **120,** 3073-3082.

Bober, E., Franz, T., Arnold, H.-H., Gruss, P. and Tremblay, P. (1994b). Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development* **120,** 603-612.

Bonner, P. H. (1978). Nerve-dependent changes in clonable myoblast populations. *Dev. Biol.* **66,** 207-219.

Bonner, P. H. and Hauschka, S. D. (1974). Clonal analysis of vertebrate myogenesis: I Early developmental events in the chick limb. *Dev. Biol.* **37,** 317-328.

Bowerman, B., Draper, B. W., Mello, C. C. and Priess, J. R. (1993). The maternal gene *skn-1* encodes a protein that is distributed unequally in early *c. elegans* embryos. *Cell* **74,** 443-452.

Bowerman, B., Eaton, B. A. and Priess, J. R. (1992). *skn-1*, a maternally expressed gene required to specify the fate of ventral blastomeres in the early *c. elegans* embryo. *Cell* **68,** 1061-1075.

Brand-Saberi, B., Ebensperger, C., Wilting, J., Balling, R. and Christ, B. (1993). The ventralizing effect of the notochord on somite differentiation in chick embryos. *Anat. Embryol.* **188,** 239-245.

Brand-Saberi, B., Muller, T. S., Wilting, J., Christ, B. and Birchmeier, C. (1996). Scatter factor/hepatocyte growth factor (SF/HGF) induces emigration of myogenic cells at interlimb level in vivo. *Dev. Biol.* **179,** 303-308.

Braun, T. and Arnold, H. H. (1996). Myf-5 and myoD genes are activated in distinct mesenchymal stem cells and determine different skeletal muscle cell lineages. *Embo J.* **15,** 310-318.

Braun, T., Bober, E., Buschhausen-Denker, G., Kohtz, S., Grzeschik, K. H. and Arnold, H. H. (1989a). Differential expression of myogenic determination genes in muscle cells: possible autoactivation by the Myf gene products. *Embo J.* **8,** 3617-3625.

Braun, T., Bober, E., Rudnicki, M. A., Jaenisch, R. and Arnold, H. H. (1994). MyoD expression marks the onset of skeletal myogenesis in Myf-5 mutant mice. *Development* **120,** 3083-3092.

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E. and Arnold, H.-H. (1989b). A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *Embo J.* **8,** 701-709.

Braun, T., Rudnicki, M., Arnold, H. and Jaenisch, R. (1992). Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell* **71,** 369-382.

Buffinger, N. and Stockdale, F. E. (1994). Myogenic specification in somites: induction by axial structures. *Development* **120,** 1443-1452.

Buffinger, N. and Stockdale, F. E. (1995). Myogenic specification of somites is mediated by diffusible factors. *Dev. Biol.* **169,** 96-108.

Carlson, J. and Carlson, B. (1993). Personal communication.

Carroll, S. B. (1995). Homeotic genes and the evolution of arthropods and chordates. *Nature* **376,** 479-485.

Chen, C. M., Kraut, N., Groudine, M. and Weintraub, H. (1996). I-mf, a novel myogenic repressor, interacts with members of the MyoD family. *Cell* **86,** 731-741.

Chen, L., Krause, M., Draper, B., Weintraub, H. and Fire, A. (1992). Body-wall muscle formation in Caenorhabditis elegans embryos that lack the MyoD homolog hlh-1. *Science* **256,** 240-243.

Cheney, C. M. and Lash, J. W. (1981). Diversification within embryonic chick somites: differential response to notochord. *Dev. Biol.* **81,** 288-298.

Cheng, T. C., Wallace, M. C., Merlie, J. P. and Olson, E. N. (1993). Separable regulatory elements governing myogenin transcription in mouse embryogenesis. *Science* **261,** 215-218.

Chevallier, A., Kieny, M. and Mauger, A. (1977). Limb-somite relationship: Origin of the limb musculature. *J. Embryol. Exp. Morph.* **41,** 245-258.

Chevallier, A., Kieny, M. and Mauger, A. (1978). Limb-somite relationship: effect of removal of somitic mesoderm on the wing musculature. *J. Embryol. exp. Morph.* **43,** 263-278.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., L., C. J., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking *Sonic hedgehog* gene function. *Nature* **383,** 407-413.

Chiquet-Ehrismann, R. (1991). Anti-adhesive molecules of the extracellular matrix. *Curr. Op. Cell Biol.* **3,** 800-804.

Choi, J., Costa, M. L., Mermelstein, C. S., Chagas, C., Holtzer, S. and Holtzer, H. (1990). MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes. *PNAS, USA* **87,** 7988-7992.

Choi, J., Schultheiss, T., Lu, M., Wachtler, F., Kuruc, N., Franke, W. W., Bader, D., Fischman, D. A. and Holtzer, H. (1989). Founder cells for the cardiac and skeletal myogenic lineages. In Cellular and molecular biology of muscle development (ed. L. H. K. a. F. E. Stockdale), pp. Alan R. Liss, Inc, New York.

Christ, B. (1969). Die knorpelentstehung in den wirbelanlagen. Experimentelle untersuchungen an huhnerembryonen. *Z. Anat. Entw. Gesch.* **129,** 177-194.

Christ, B., Brand-Saberi, B., Grim, M. and Wilting, J. (1992). Local signalling in dermomyotomal cell type specification. *Anat. Embryol.* **186,** 505-510.

Christ, B., Jacob, H. and Jacob, M. (1974). Uber den ursprung der flugelmuskulature. *Experientia* **30,** 1446-1448.

Christ, B., Jacob, H. and Jacob, M. (1977). Experimental analysis of the origin of the wing musculature in avian embryos. *Anat. Embryol.* **150,** 171-186.

Christ, B., Jacob, M. and Jacob, H. J. (1983). On the origin and development of the ventrolateral abdominal muscles in the avian embryo. An experimental and ultrastructural study. *Anat. Embryol.* **166,** 87-101.

Cohn, M. J., Izpisua-Belmonte, J. C., Abud, H., Heath, J. K. and Tickle, C. (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* **80,** 739-746.

Colamarino, S. and Tessier-Lavigne, M. (1995). The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. *Cell* **81,** 621-629.

Conklin, E. G. (1905). Mosaic development in ascidian eggs. *J. Exp. Zool.* **2,** 285-306.

Constantinides, P. G., Jones, P. A. and Gevers, W. (1977). Functional striated muscle cells from non-myoblast precursors following 5-azacytidine treatment. *Nature* **267,** 364-366.

Corbin, V., Michelson, A. M., Abmayr, S. M., Neel, V., Alcamo, E., Maniatis, T. and Young, M. W. (1991). A role for the *Drosophila* neurogenic genes in mesoderm differentiation. *Cell* **67,** 311-323.

Cossu, G., Kelly, R., Tajbakhsh, S., Di Donna, S., Vivarelli, E. and Buckingham, M. (1996). Activation of different myogenic pathways: myf-5 is induced by the neural tube and MyoD by the dorsal ectoderm in mouse paraxial mesoderm. *Development* **122,** 429-37.

Couly, G. F., Colty, P. M. and Le Douarin, N. L. (1992). The developmental fate of the cephalic mesoderm in quail-chick chimeras. *Development* **114,** 1-15.

Cox, K. H., DeLeon, D. V., Angerer, L. M. and Angerer, R. C. (1984). Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. *Dev. Biol.* **101,** 485-502.

Crossley, P. H. and Martin, G. R. (1995). The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121,** 439-51.

Crossley, P. H., Minowada, G., MacArthur, C. A. and Martin, G. R. (1996). Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell* **84,** 127-36.

Cserjesi, P., Lilly, B., Bryson, L., Wang, Y., Sassoon, D. A. and Olson, E. N. (1992). MHox: a mesodermally restricted homeodomain protein that binds an essential site in the muscle creatine kinase enhancer. *Development* **115,** 1087-1101.

Cunha, G. R. (1976a). Epithelial-stromal interactions in the development of the urogenital tract. *Int. Rev. Cytol.* **47,** 137-149.

Cunha, G. R. (1976b). Stromal induction and specification of morphogenesis and cytodifferentiation of the epithelia of the Mullerian ducts and urogenital sinus during development of the uterus and vagina in mice. *J. Exp. Zool.* **196,** 361-370.

Currie, P. D. and Ingham, P. W. (1996). Induction of a specific muscle cell type by a hedgehog-like protein in zebrafish. *Nature* **382,** 452-455.

Daston, G., Lamar, F., Olivier, M. and Goulding, M. (1996). Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse. *Development* **122,** 1017-1027.

Davis, R., Weintraub, H. and Lassar, A. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51,** 987-1000.

Davis, R. L., Cheng, P. F., Lassar, A. B. and Weintraub, H. (1990). The myoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell* **60,** 733-746.

Davis, R. L. and Weintraub, H. (1992). Acquisition of myogenic specificity by replacement of three amino acid residues from MyoD into E12. *Science* **256,** 1027-1030.

de la Brousse, C. and Emerson, C. (1990). Localized expression of a myogenic regulatory gene, qmf1, in the somite dermatome of avian embryos. *Genes Dev.* **4,** 567-581.

deLapeyriere, O., Ollendorff, V., Planceh, J., Ott, M.-O., Pizette, S., Coulier, F. and Birnbaum, D. (1993). Expression of the Fgf6 gene is restricted to developing skeletal muscle in the mouse embryo. *Development* **118,** 601-611.

Denetclaw, W. F., Christ, B. and Ordahl, C. P. (1996). Location and growth of epaxial myotome precursor cells. *Development* **in press,**

Detwiler (1955). *J. Exp. Zool.* **129,** 45-76.

Devlin, B. H. and Konigsberg, I. R. (1983). Re-entry into the cell cycle of differentiated skeletal myocytes. *Dev. Biol.* **95,** 175-192.

Devlin, R. B. and Emerson, C. P. J. (1978). Coordinate regulation of contractile protein synthesis during myoblast differentiation. *Cell* **13,** 599-611.

Devlin, R. B. and Emerson, C. P. J. (1979). Coordinate accumulation of contractile protein mRNAs during myoblast differentiation. *Dev. Biol.* **69,** 202-216.

Devoto, S. H., Melancon, E., Eisen, J. S. and Westerfield, M. (1996). Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. *Development* **122,** 3371-3380.

Dienstman, S. R., Biehl, J., Holtzer, S. and Holtzer, H. (1974). Myogenic and chondrogenic lineages in developing limb buds grown in in vitro. *Dev. Biol.* **39,** 83-95.

Dietrich, S. and Gruss, P. (1995). undulated phenotypes suggest a role of Pax-1 for the development of vertebral and extravertebral structures. *Dev. Biol.* **167,** 529-548.

Driesch, H. (1893). Sur Verlagerung der Blastomeren des Echinideneies. *Anat. Anz.* **8,** 348-357.

Driever, W. and Nusslein-Volhard, C. (1988). A gradient of bicoid protein in *Drosophila* embryos. *Cell* **54,** 83-93.

Duband, J. L., Dufour, S., Hatta, K., Takeichi, M., Edelman, G. and Thiery, J. P. (1987). Adhesion molecules during somitogenesis in the avian embryo. *J. Cell Biol.* **104,** 1361-1374.

Duprez, D. M., Coltey, M., Amthor, H., Brickell, P. M. and Tickle, C. (1996). Bone morphogenetic protein-2 (BMP-2) inhibits muscle development and promotes cartilage formation in chick limb bud cultures. *Dev. Biol.* **174,** 448-452.

Edmondson, D. G., Cheng, T. C., Cserjesi, P., Chakraborty, T. and Olson, E. N. (1992). Analysis of the myogenin promoter reveals an indirect pathway for positive autoregulation mediated by the muscle-specific enhancer factor MEF-2. *Mol. Cell Biol.* **12,** 3665-3677.

Edmondson, D. G. and Olson, E. N. (1993). Helix-loop-helix proteins as regulators of muscle-specific transcription. *J. Biol. Chem.* **268,** 755-758.

Emerson, C. (1993). Skeletal myogenesis: genetics and embryology to the fore. *Current Opinion in Genetics and Development* **3,** 265-274.

Epstein, D., Vekemans, M. and Gros, P. (1991). *Splotch (Sp2H)*, a mutation affecting development of the mouse neural tube, shows a deletion within the paired homeodomain of Pax-3. *Cell* **67,** 767-774.

Epstein, J. A., Lam, P., Jepeal, L., Maas, R. L. and Shapiro, D. N. (1995). Pax3 inhibits myogenic differentiation of cultured myoblast cells. *J. Biol. Chem.* **270,** 11719-11722.

Epstein, J. A., Shapiro, D. N., Cheng, J., Lam, P. Y. and Maas, R. I. (1996). Pax3 modulates expression of the c-Met receptor during limb muscle development. *Proc. Natl. Acad. Sci. USA* **93,** 4213-4218.

Eyal-Giladi, H., Debby, A. and Harel, N. (1992). The posterior section of the chick's area pellucida and its involvement in hypoblast and primitive streak formation. *Development* **116,** 819-830.

Faerman, A., Pearson-White, S., Emerson, C. P. J. and Shani, M. (1993). Ectopic expression of MyoD1 in mice causes prenatal lethalities. *Dev. Dynam.* **196,** 165-173.

Fan, C.-M., Porter, J. A., Chiang, C., Chang, D. T., Beachy, P. A. and Tessier-Lavigne, M. (1995). Long-range sclerotome induction by sonic hedgehog: Direct role of the amino-terminal cleavage product and modulation by the cyclic AMP signaling pathway. *Cell* **81,** 457-465.

Fan, C.-M. and Tessier-Lavigne, M. (1994). Patterning of mammalian somites by surface ectoderm and notochord: Evidence for sclerotome induction by a hedgehog homolog. *Cell* **79,** 1175-1186.

Filvaroff, E. and Derynck, R. (1996). Induction of myogenesis in mesenchymal cells by MyoD depends on their degree of differentiation. *Dev. Biol.* **178,** 459-471.

Franz, T. (1993). The Splotch (Sp^{1H}) and Splotch-delayed (Sp^{d}) alleles: differential phenotypic effects on neural crest and limb musculature. *Anat. Embryol.* **187,** 371-377.

Franz, T., Kothary, R., Surani, M. A. H., Halata, Z. and Grim, M. (1993). The *Splotch* mutation interferes with muscle development in the limbs. *Anat. Embryol.* **187,** 153-160.

Frohman, M. A., Boyle, M. and Martin, G. R. (1990). Isolation of the mouse *Hox-2.9* gene; analysis of embryonic expression suggests that positional information along the anterior-posterior axis is specified by mesoderm. *Development* **110,** 589-607.

Fuchtbauer, E. M. (1995). Expression of M-twist during postimplantation development of the mouse. *Dev. Dyn.* **204,** 316-22.

Gamel, A. J., Brand-Saberi, B. and B., C. (1995). Halves of epithelial somites and segmental plate show distinct muscle differentiation behavior in vitro compared to entire somites and segmental plate. *Dev. Biol.* **172,** 625-639.

George-Weinstein, M., Gerhart, J., Foti, G. and Lash, J. (1994). Maturation of myogenic and chondrogenic cells in the presomitic mesoderm of the chick embryo. *Exp. Cell Res.* **211,** 263-274.

George-Weinstein, M., Gerhart, J., Reed, R., Flynn, J., Callihan, B., Mattiacci, M., Miehle, C., Foti, G., Lash, J. and Weintraub, H. (1996). Skeletal myogenesis: the preferred pathway of chick embryo epiblast cells in vitro. *Dev. Biol.* **173,** 279-291.

Gilbert, S. F. (1994). Developmental Biology, 4th ed. Sinauer Associates Inc., Sunderland, MA.

Goldhamer, D. J., Brunk, B. P., Faerman, A., King, A., Shani, M. and Emerson, C. P. J. (1995). Embryonic activation of the myoD gene is regulated by a highly conserved distal control element. *Development* **121,** 637-649.

Goldhamer, D. J., Faerman, A., Shani, M. and Emerson, C. P. J. (1992). Regulatory elements that control the lineage-specific expression of myoD. *Science* **256,** 538-542.

Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A. and Scott, M. P. (1996). Conservation of the Hedgehog/patched signalling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev.* **10,** 301-312.

Gordon, J. S. and Lash, J. W. (1974). *In vitro* chondrogenesis and cell viability. *Dev. Biol.* **36,** 88-104.

Gossett, L. A., Kelvin, D. J., Sternberg, E. A. and Olson, E. N. (1989). A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. *Mol. Cell. Biol.* **9,** 5022-5033.

Goulding, M., Lumsden, A. and Paquette, A. J. (1994). Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. *Development* **120,** 957-971.

Goulding, M., Sterrer, S., Fleming, J., Balling, R., Nadeau, J., Moore, K., Brown, S., Steel, K. and Gruss, P. (1993a). Analysis of the Pax-3 gene in the mouse mutant *Splotch*. *Genomics* **17,** 355-363.

Goulding, M. D., Chalepakis, G., Deutsch, U., Erselius, J. R. and Gruss, P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. *Embo J.* **10,** 1135-1147.

Goulding, M. D., Lumsden, A. and Gruss, P. (1993b). Signals from the notochord and floor plate regulate the region-specific expression of two Pax genes in the developing spinal cord. *Development* **117,** 1001-1016.

Grainger, R. M. (1992). Embryonic lens induction: Shedding light on vertebrate tissue determination. *Trends Genet.* **8,** 349-355.

Grass, S., Arnold, H. H. and Braun, T. (1996). Alterations in somite patterning of Myf-5-deficient mice: a possible role for FGF-4 and FGF-6. *Development* **122,** 141-150.

Green, J. B. A. and Smith, J. C. (1990). Graded changes in dose of a Xenopus activin A homologue elicit stepwise transitions in embryonic cell fate. *Nature* **347,** 391-394.

Greenwald, I. and Rubin, G. (1992). Making a difference: The role of cell-cell interactions in establishing separate identities for equivalent cells. *Cell* **68,** 271-281.

Grobstein, C. (1955). Induction interaction in the development of the mouse metanephros. *J. Exp. Zool.* **130,** 319-340.

Grobstein, C. (1956). Trans-filter induction of tubules in mouse metanephrogenic mesenchyme. *Exp. Cell Res.* **10,** 424-440.

Grobstein, C. and Holtzer, H. (1955). In vitro studies of cartilage induction in mouse somite mesoderm. *J. Exp. Zool.* **128,** 333-359.

Gruss, P. and Walther, C. (1992). Pax in development. *Cell* **69,** 719-722.

Gurdon, J. B. (1962). The developmental capacity of nuclei taken from intestinal epithelial cells of feeding tadpoles. *J. Embryol. Exp. Morph.* **10,** 622-640.

Gurdon, J. B. (1987). Embryonic induction: Molecular prospects. *Development* **99,** 285-306.

Gurdon, J. B. (1988). A community effect in animal development. *Nature* **336,** 772-774.

Gurdon, J. B., Harger, P., Mitchell, A. and Lemaire, P. (1994). Activin signalling and response to a morphogen gradient. *Nature* **371,** 487-492.

Halder, G., Callaerts, P. and Gehring, W. J. (1995). Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila. Science* **267,** 1788-1792.

Halpern, M. E., Ho, R. K., Walker, R. C. and Kimmel, C. B. (1993). Induction of muscle pioneers and floor plate is distinguished by the zebrafish *no tail* mutation. *Cell* **75,** 99-111.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 49-92.

Hamburger, V. and Hamilton, H. L. (1992). A series of normal stages in the development of the chick embryo. *Developmental Dynamics* **195,** 231-272.

Hamilton, L. (1969). The formation of somites in *Xenopus. J. Exp. Emb. Morph.* **22,** 253-264.

Hammerschmidt, M., Bitgood, M. J. and McMahon, A. P. (1996). Protein kinase A is a common negative regulator of Hedgehog signaling in the vertebrate embryo. *Genes Dev.* **10,** 647-658.

Hartenstein, A. Y., Rugendorff, A., Tepass, U. and Hartenstein, V. (1992). The function of the neurogenic genes during epithelial development in the *Drosophila* embryo. *Development* **116,** 1203-1220.

Harvey, R. (1990). The Xenopus myoD gene: an unlocalized maternal mRNA predates lineage-restricted expression in the early embryo. *Development* **108,** 669-680.

Hasty, P., Bradley, A., Morris, J., Edmondson, D., Venuti, J., Olson, E. and Klein, W. (1993). Muscle deficiency and neonatal death in mice with a targeted mutaion in the myogenin gene. *Nature* **364,** 501-506.

Hebrok, M., Wertz, K. and Fuchtbauer, E.-M. (1994). M-twist is an inhibitor of muscle differentiation. *Dev. Biol.* **165,** 537-544.

Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of *Drosophila. Cell* **64,** 1083-1092.

Hollenberg, S. M., Cheng, P. F. and Weintraub, H. (1993). Use of a conditional MyoD transcription factor in studies of MyoD trans-activation and muscle determination. *Proc. Natl. Acad. Sci. USA* **90,** 8028-8032.

Holley, S. A., Jackson, P. D., Sasai, Y., Lu, B., De Robertis, E., Hoffmann, F. M. and Ferguson, E. L. (1995). A conserved system for dorsal-ventral patterning in insects and vertebrates involving *sog* and *chordin*. *Nature* **376,** 249-253.

Holtzer, H. (1978). Cell lineages, stem cells and the "quantal" cell cycle concept. In Stem Cells and Tissue Homeostasis (ed. B. I. Lord, C. S. Potten and R. J. Cole), pp. 1-27. Cambridge University Press,

Holtzer, H. and Detwiler, S. R. (1953). An experimental analysis of the development of the spinal column. *J. Exp. Zool.* **123,** 335-369.

Holtzer, H., DiIullo, C., Costa, M. L., Lu, M., Choi, J., Mermelstein, C. S., Schultheiss, T. and Holtzer, S. (1991). Striated myoblasts and multinucleated myotubes induced in non-muscle cells by MyoD are similar to normal in vivo and in vitro counterparts. In *Frontiers in Muscle Research* (ed. E. Ozawa, T. Masaki and Y. Nabeshima), pp. 187-207. Elsevier Science Publishers B. V., Amsterdam.

Holtzer, H., Marshall, J. and Finck, H. (1957). An analysis of myogenesis by use of fluorescent anti-myosin. *J. Biophys. Biochem. Cytol.* **3,** 705.

Hopwood, N. D. and Gurdon, J. B. (1990). Activation of muscle genes without myogenesis by ectopic expression of myoD in frog embryo cells. *Nature* **347,** 197-200.

Hopwood, N. D., Pluck, A., Gurdon, J. B. and Dilworth, S. M. (1992). Expression of XMyoD protein in early Xenopus laevis embryos. *Development* **114,** 31-38.

Horstadius, S. (1939). The mechanics of sea urchin development studied by operative methods. *Biol. Rev.* **14,** 132-179.

Jacob, F. and Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* **3,** 318-356.

Jacobson, A. G. (1966). Inductive processes in embryonic development. *Science* **152,** 25-34.

Johnson, R. L., Laufer, E., Riddle, R. D. and Tabin, C. (1994). Ectopic expression of Sonic hedgehog alters dorso-ventral patterning of somites. *Cell* **79,** 1165-1173.

Johnston, M. C., Noden, D. M., Hazelton, R. D., Coulombre, J. L. and Coulombre, A. J. (1979). Origins of avian ocular and periocular tissues. *Experimental Eye Research* **29,** 27-43.

Jones, F. S., Chalepakis, G., Gruss, P. and Edelman, G. M. (1992a). Activation of the cytotactin promoter by the homeobox-containing gene *Evx-1*. *Proc. Natl. Acad. Sci. USA* **89,** 2091-2095.

Jones, F. S., Prediger, E. A., Bittner, D. A., DeRobertis, E. M. and Edelman, G. M. (1992b). Cell adhesion molecules as targets for *Hox* genes: Neural cell adhesion molecule promoter activity is modulated by cotransfection with *Hox-2.5* and *-2.4*. *Proc. Natl. Acad. Sci. USA* **89,** 2086-2090.

Kaehn, K., Jacob, H. J., Christ , B., Hinrichsen, K. and Poelmann, R. E. (1988). The onset of myotome formation in the chick. *Anat. Embryol.* **177,** 191-201.

Kato, K. and Gurdon, J. B. (1993). Single-cell transplantation determines the time when *Xenopus* muscle precursor cells acquire a capacity for autonomous differentiation. *Proc. Natl. Acad. Sci. USA* **90,** 1310-1314.

Kelly, A. M. and Rubinstein, N. A. (1980). Why are fetal muscles slow? *Nature* **288,** 266-269.

Kennedy, T. F., Serafini, T., de la Torre, J. R. and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* **78,** 425-435.

Kenny-Mobbs, T. and Thorogood, P. (1987). Autonomy of differentiation in avian brachial somites and the influence of adjacent tissues. *Development* **100,** 449-462.

Keynes, R. and Stern, C. (1988). Mechanisms of vertebrate segmentation. *Development* **103,** 413-429.

Kieny, M. (1980). The concept of a myogenic cell line in developing avian limb buds. In Teratology of the limbs (ed. pp. 79-88. Walter de Gruyter and Co., Berlin, New York.

Kieny, M., Pautou, M.-P. and Chevallier, A. (1981). On the stability of the myogenic cell line in avian limb bud development. *Arch. d'Anat. Micro.* **70,** 81-90.

Konieczny, S. F. and Emerson, C. P. J. (1984). 5-Azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: evidence for regulatory genes controlling determination. *Cell* **38,** 791-800.

Konigsberg, I. R. (1963). Clonal analysis of myogenesis. *Science* **140,** 1273-1284.

Kopan, R., Nye, J. S. and Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development* **120,** 2385-2396.

Krause, M., Fire, A., Harrison, S. W., Priess, J. and Weintraub, H. (1990). CeMyoD accumulation defines the body wall muscle cell fate during C. elegans embryogenesis. *Cell* **63,** 907-919.

Krenn, V., Gorka, P., Wachtler, F., Christ, B. and Jacob, H. (1988). On the origin of cells determined to form skeletal muscle in avian embryos. *Anat. Embryol.* **179,** 49-54.

Lash, J., Holtzer, S. and Holtzer, H. (1957). An experimental analysis of the development of the spinal column. VI Aspects of cartilage induction. *Exp. Cell Res.* **13,** 292-303.

Lash, J. W. (1967). Differential behavior of anterior and posterior embryonic chick somites in vitro. *J. Exp. Zool.* **165,** 47-56.

Lassar, A. B., Buskin, J. N., Lockshon, D., Davis, R. L., Apone, S., Hauschka, S. D. and Weintraub, H. (1989). MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. *Cell* **58,** 823-831.

Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D. and Weintraub, H. (1991). Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell* **66,** 305-15.

Lassar, A. B., Patterson, B. M. and Weintraub, H. (1986). Transfection of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts. *Cell* **47,** 649-656.

Le Douarin, N. (1973). A Feulgen-positive nucleolus. *Exp. Cell Res.* **77,** 459-468.

Le Douarin, N. and McLaren, A. (1984). Chimeras in Developmental Biology. In (ed. pp. Academic Press, Inc., Orlando, FL 32887.

Le Douarin, N. M., Houssaint, E., Jotereau, F. V. and Belo, M. (1975). Origin of hemopoietic stem cells in embryonic bursa of Fabricius and bone marrow studied through interspecific chimeras. *Proc. Natl. Acad. Sci. USA* **72,** 2701-2705.

Le Douarin, N. M. and Teillet, M. A. (1974). Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neuroectodermal mesenchymal derivatives, using a biological cell marking technique. *Dev. Biol.* **41,** 162-184.

Lewis, W. H. and Lewis, M. R. (1917). Behavior of cross striated muscle in tissue cultures. *J. Anat.* **22,** 169-194.

Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M. P. and Olson, E. N. (1992). FGF inactivates myogenic helix-loop-helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains. *Cell* **71,** 1181-1194.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B. M., Schulz, R. A. and Olson, E. N. (1995). Requirement of MADS domain transcription factor D-MEF2 for muscle formation in Drosophila. *Science* **267,** 688-693.

Lin, Z., Dechesne, C. A., Eldridge, J. and Paterson, B. M. (1989). An avian muscle factor related to MyoD1 activates muscle-specific promoters in nonmuscle cells of different germ-layer origin and in BrdU-treated myoblasts. *Genes Dev.* **3,** 986-996.

Lin-Jones, J. and Hauschka, S. D. (1996). Myogenic determination factor expression in the developing avian limb bud: an RT-PCR analysis. *Dev. Biol.* **174,** 407-422.

MacCabe, J. A., Errick, J. and Saunders, J. W. J. (1974). Ectodermal control of the dorsoventral axis in the leg bud of the chick embryo. *Dev. Biol.* **39,** 69-82.

Mansouri, A., Stoykova, A., Torres, M. and Gruss, P. (1996). Dysgenesis of cephalic neural crest derivatives in Pax7 -/- mutant mice. *Development* **122,** 831-838.

Mar, J. H. and Ordahl, C. P. (1988). A conserved CATTCCT motif is required for skeletal muscle-specific activity of the cardiac troponin T gene promoter. *Proc. Nat. Acad. Sci. USA* **85,** 6404-6408.

Mar, J. H. and Ordahl, C. P. (1990). M-CAT binding factor, a novel trans-acting factor governing muscle-specific transcription. *Mol. Cell. Biol.* **10,** 4271-4283.

Marcelle, C., Wolf, J. and Bronner-Fraser, M. (1995). The in vivo expression of FGF receptor FREK mRNA in avian myoblasts suggests a role in muscle growth and differentiation. *Dev. Biol.* **172,** 100-114.

Mauger, A. and Kieny, M. (1980). Migratory and organogenetic capacities of muscle cells in bird embryos. *Wilhelm Roux's Arch.* **189,** 123-134.

Mauger, A., Kieny, M. and Chevallier, A. (1980). Limb-somite relationship: myogenic potentialities of somatopleural mesoderm. *Arch. d'Anat. Micro.* **69,** 175-195.

Meedel, T. H., Crowthier, R. J. and Whittaker, J. R. (1987). Determinative properties of muscle lineages in ascidian embryos. *Development* **100,** 245-260.

Menko, A. S. and Boettiger, D. (1987). Occupation of the extracellular matrix integrin is a control point for myogenic differentiation. *Cell* **51,** 51-57.

Michelson, A. M., Abmayr, S. M., Bate, M., Arias, A. M. and Maniatis, T. (1990). Expression of a MyoD family member prefigures muscle pattern in *Drosophila* embryos. *Genes Dev.* **4,** 2086-2097.

Milaire, J. (1976). Contribution cellulaire des somites a la genese des bourgeons de membres posterieurs chez la souris. *Arch Biol (Bruxelles)* **87,** 315-343.

Miller, J. B. and Stockdale, F. E. (1986). Developmental origins of skeletal muscle fibers: clonal analysis of myogenic cell lineages based on expression of fast and slow myosin heavy chains. *Proc. Nat. Acad. Sci. USA* **83,** 3860-3864.

Miner, J. H., Miller, J. B. and Wold, B. (1992). Skeletal muscle phenotypes initiated by ectopic MyoD in transgenic mouse heart. *Development* **114,** 853-860.

Miner, J. H. and Wold, B. (1990). Herculin, a fourth member of the *MyoD* family of myogenic regulatory genes. *Proc. Nat. Acad. Sci., USA* **87,** 1089-1093.

Mintz, B. and Baker, W. W. (1967). Normal mammalian muscle differentiation and gene control of isocitrate dehydrogenase synthesis. *Proc. Natl. Acad. Sci. USA* **58,** 592-598.

Moase, C. E. and Trasler, D. G. (1990). Delayed neural crest cell emigration from Sp and Sp^d mouse neural tube explants. *Teratology* **42,** 171-182.

Moase, C. E. and Trasler, D. G. (1991). N-CAM alteration in splotch neural tube defect mouse embryos. *Development* **113,** 1049-1058.

Molkentin, J. D., Black, B. L., Martin, J. F. and Olson, E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell* **83,** 1125-1136.

Molkentin, J. D., Black, B. L., Martin, J. F. and Olson, E. N. (1996a). Mutational analysis of the DNA binding, dimerization, and transcriptional activation domains of MEF2C. *Mol. Cell Biol.* **16,** 2627-2636.

Molkentin, J. D., Li, L. and Olson, E. N. (1996b). Phosphorylation of the MADS-box transcription factor MEF2C enhances its DNA binding activity. *J. Biol. Chem.* **271,** 17199-17204.

Molkentin, J. D. and Olson, E. N. (1996). Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. *Proc. Natl. Acad. Sci. USA* **93,** 9366-9373.

Morgan, B. A., Izpisua-Belmonte, J. C., Duboule, D. and Tabin, C. J. (1992). Targeted misexpression of Hox-4.6 in the avian limb bud causes apparent homeotic transformations. *Nature* **358,** 236-239.

Munsterberg, A. E., Kitajewski, J., Bumcrot, D. A., McMahon, A. P. and Lassar, A. B. (1995). Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev.* **9,** 2911-2922.

Munsterberg, A. E. and Lassar, A. B. (1995). Combinatorial signals from the neural tube, floorplate and notochord induce myogenic bHLH gene expression in the somite. *Development* **121,** 651-660.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I. and Nabeshima, Y. (1993). Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature* **364,** 532-535.

Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85,** 357-368.

New, D. A. T. (1955). A new technique for the cultivation of the chick embryo *in vitro*. *J. Embryol. Exp. Morph.* **3,** 326-331.

Newman, S. A., Pautou, M.-P. and Kieny, M. (1981). The distal boundary of myogenic primordia in chimeric avian limb buds and its relation to an accessible population of cartilage progenitor cells. *Dev. Biol.* **84,** 440-448.

Nieuwkoop, P. D. (1969). The formation of the mesoderm in urodele amphibians. I. Induction by the endoderm. *Wilhelm Roux Arch. Entwicklungsmech. Org.* **162,** 341-373.

Nieuwkoop, P. D. and Nigtevecht, G. V. (1954). Neural activation and transformation in explants of competent ectoderm under the influence of fragments of anterior notochord in urodeles. **2,** 175-193.

Niswander, L. and Martin, G. (1992). Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* **114,** 755-768.

Noden, D. M. (1983). The embryonic origins of avian cephalic and cervical muscle and associated connective tissues. *Amer. J. Anat.* **168,** 257-276.

Noden, D. M. (1984). The use of chimeras in analyses of craniofacial development. In Chimeras in Developmental Biology (ed. N. Le Douarin and A. McLaren), pp. 241-280. Academic Press, London.

Olson, E. (1992). Interplay between proliferation and differentiation within the myogenic lineage. *Dev Biol* **154,** 261-272.

Ordahl, C. (1992). Developmental Regulation of Sarcomeric Gene Expression. In Current Topics in Developmental Biology (ed. E. Bearer), pp. 145-168. Academic Press, New York.

Ordahl, C. P. (1993). Myogenic lineages within the developing somite. In Molecular Basis of Morphogenesis (ed. M. Bernfield), pp. John Wiley & Sons, New York.

Ordahl, C. P. and Christ, B. (1996). Avian somite transplantation: A review of basic methods. In *Methods in Cell Biology* (ed. C. P. J. Emerson), pp. Academic Press, San Diego.

Ordahl, C. P. and Le Douarin, N. (1992). Two myogenic lineages within the developing somite. *Development* **114,** 339-353.

Ott, M., Bober, E., Lyons, G., Arnold, H. and Buckingham, M. (1991). Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. *Development* **111,** 1097-1107.

Pain, B., Clark, M. E., Shen, M., Nakazawa, H., Sakurai, M., Samarut, J. and Etches, R. J. (1996). Longterm in vitro culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities. *Development* **122,** 2339-2348.

Patapoutian, A., Yoon, J. K., Miner, J. H., Wang, S., Stark, K. and Wold, B. (1995). Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. *Development* **121,** 3347-3358.

Paterson, B. and Strohman, R. (1972). Myosin synthesis in cultures of differentiating chicken embryo skeletal muscle. *Dev. Biol.* **29,** 113-138.

Paterson, B. M., Walldorf, U., Eldridge, J., Dubendorfer, A., Frasch, M. and Gehring, W. J. (1991). The *Drosophila* homologue of vertebrate myogenic-determination genes encodes a transiently expressed nuclear protein marking primary myogenic cells. *Proc. Natl. Acad. Sci. USA* **88,** 3782-3786.

Pinney, D. F., Pearson-White, S. H., Konieczny, S. F., Latham, K. E. and Emerson, C. P. J. (1988). Myogenic lineage determination and differentiation: evidence for a regulatory gene pathway. *Cell* **53,** 781-793.

Placzek, M., Tessier-Lavigne, M., Yamada, T., Jessell, T. and Dodd, J. (1990). Mesodermal control of neural cell identity: floor plate induction by the notochord. *Science* **250,** 985-988.

Pourquie, O., Coultey, M., Teillet, M.-A., Ordahl, C. P. and Le Douarin, N. M. (1993). Control of dorso-ventral patterning of the somitic derivatives by notochord and floor plate. *Proc. Natl. Acad. Sci. USA* **90,** 5242-5246.

Pourquie, O., Fan, C. M., Coltey, M., Hirsinger, F., Watanabe, Y., Breant, C., Francis-West, P., Brickell, P., Tessier-Lavigne, M. and Le Douarin, N. M. (1996). Lateral and axial signals involved in avian somite patterning: a role for BMP4. *Cell* **84,** 461-471.

Pownall, M. E. and Emerson, C. P. (1992). Sequential activation of three myogenic regulatory genes during somite morphogenesis in quail embryos. *Dev. Biol.* **151,**

Pownall, M. E., Strunk, K. E. and Emerson, C. P. J. (1996). Notochord signals control the transcriptional cascade of myogenic bHLH genes in somites of quail embryos. *Development* **122,** 1475-1488.

Rabl, C. (1888). Ueber die differenzierung des mesoderms. *Verh. anat. Ges., Wurzburg* **2,** 140-146.

Rawls, A., Morris, J. H., Rudnicki, M. A., Braun, T., Arnold, H. H., Klein, W. H. and Olson, E. N. (1995). Myogenin's functions do not overlap with those of MyoD or Myf5 during mouse embryogenesis. *Dev. Biol.* **172,** 37-50.

Reaume, A. G., Conlon, R. A., Zirngibl, R., Yamaguchi, T. P. and Rossant, J. (1992). Expression analysis of a *Notch* homologue in the mouse embryo. *Dev. Biol.* **154,** 377-387.

Reilly, K. M. and Melton, D. A. (1996). Short-range signaling by candidate morphogens of the TGF beta family and evidence for a relay mechanism of induction. *Cell* **86,** 743-754.

Remak, R. (1855). Untersuchungen uber die Entwicklung der Wirbeltiere. Reimer, Berlin.

Riddle, R. D., Fosini, M., Nelson, C., Tsuchida, T., Jessell, T. M. and Tabin, C. J. (1995). Induction of the LIM homeobox gene Lmx1 by WNT7a establishes dorsoventral pattern in the vertebrate limb. *Cell* **83,** 631-640.

Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75,** 1401-1416.

Rong, P. M., Teillet, M.-A., Ziller, C. and Le Douarin, N. M. (1992). The neural tube/notochord complex is necessary for vertebral but not limb and body wall striated muscle differentiation. *Development* **115,** 657-672.

Rosenquist, G. C. (1966). A radioautographic study of labeled grafts in the chick blastoderm. Development from primitive-streak stages to stage 12. *Carnegie Inst. Wash. Contr. Embryol.* **38,** 31-110.

Rudnicki, M., Braun, T., Hinuma, S. and Jaenisch, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell* **71,** 383-390.

Rudnicki, M. A., Schnegelsberg, P. N. J., Stead, R. H., Braun, T., Arnold, H.-H. and Jaenisch, R. (1993). MyoD or myf-5 is required for the formation of skeletal muscle. *Cell* **75,** 1351-1359.

Rupp, R. A., Snider, L. and Weintraub, H. (1994). Xenopous embryos regulate the nuclear localization of XMyoD. *Genes Dev.* **8,** 1311-1323.

Rupp, R. A. and Weintraub, H. (1991). Ubiquitous MyoD transcription at the midblastula transition precedes induction-dependent MyoD expression in presumptive mesoderm of X. laevis. *Cell* **65,** 927-937.

Rushton, E., Drysdale, R., Abmayr, S. M., Michelson, A. M. and Bate, M. (1995). Mutations in a novel gene, *myoblast city*, provide evidence in support of the founder cell hypothesis for *Drosophila* muscle development. *Development* **121,** 1979-1988.

Rutz, R., Haney, C. and Hauschka, S. (1982). Spatial analysis of limb bud myogenesis: A proximodistal gradient of muscle colony-forming cells in chick embryo leg buds. *Dev Biol* **90,** 399-411.

Sasaki, H., Jones, P. A., Chaillet, J. R., Ferguson-Smith, A. C., Barton, S. C., Reik, W. and Surani, M. A. (1992). Parental imprinting: potentially active chromatin of the represssed maternal allele of the mouse insulin-like growth factor II (IGF2) gene. *Genes Dev.* **6,** 1843-1856.

Sassoon, D., Lyons, G., Wright, W. E., Lin, V., Lassar, A., Weintraub, H. and Buckingham, M. (1989). Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. *Nature* **341,** 303-307.

Saunders, J. W. J., Gasseling, M. T. and Errick, J. E. (1976). Inductive activity and enduring cellular constitution of a supernumerary apical ectodermal ridge grafted to the limb bud of the chick embryo. *Dev. Biol.* **50,** 16-25.

Schoenwolf, G. G. (1991). Cell movements in the epiblast during gastrulation and neurulation in avian embryos. In Gastrulation: Movements, Patterns and Molecules (ed. R. Keller, W. H. Clark and F. Griffin), pp. 1-28. Plenum, New York.

Searls, R. and Janners, M. (1969). The stabilization of cartilage properties in the cartilage-forming mesenchyme of the embryonic chick limb. *J Exp Zool* **170,** 365-376.

Seed, J. and Hauschka, S. D. (1984). Temporal separation of the migration of distinct myogenic precursor populations into the developing chick wing bud. *Dev. Biol.* **106,** 389-393.

Selleck, M. and Stern, C. (1991). Fate mapping and cell lineage analysis of Hensen's node in the chick embryo. *Development* **112,** 615-626.

Selleck, M. A. and Bronner-Fraser, M. (1996). The genesis of avian neural crest cells: a classic embryonic induction. *Proc. Nat. Acad. Sci. USA* **93,** 9352-9357.

Serafini, T., Kennedy, T. F., Galko, M. J., Mirzayan, C., Jessell, T. M. and Tessier-Lavigne, M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. *Cell* **78,** 409-424.

Slack, J. M. W. (1983). From egg to embryo: Determinative events in early development. Cambridge University Press, New York.

Solursh, M. and Reiter, R. S. (1988). Inhibitory and stimulatory effects of limb ectoderm on in vitro chondrogenesis. *J. Exp. Zool.* **248,** 147-154.

Spemann, H. and Mangold, H. (1924). Induction of embryonic primordia by implantation of organizers from a different species. In *Foundations of Experimental Embryology* (ed. J. M. Oppenheimer), pp. 144-184. Hafner, New York.

Spemann, H. and Schotte, O. (1932). Uber xenoplatische transplantation als mittel zur analyse der embryonalen induction. *Naturwissenschaften* **20,** 463-467.

Spicer, D. B., Rhee, J., Cheung, W. L. and Lassar, A. B. (1996). Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. *Science* **272,** 1476-1480.

Spratt, N. T. J. (1946). Formation of the primitive streak in the explanted chick blastoderm marked with carbon particles. *J. Exp. Zool.* **103,** 259-304.

Stark, R. and Searls, R. (1974). The establishment of the cartilage pattern in the embryonic chick wing, and evidence for a role of the dorsal and ventral ectoderm in normal wing development. *Dev Biol* **38,** 51-63.

Stern, H. M., Brown, A. M. and Hauschka, S. D. (1995). Myogenesis in paraxial mesoderm: preferential induction by dorsal neural tube and by cells expressing Wnt-1. *Development* **121,** 3675-3686.

Stern, H. M. and Hauschka, S. D. (1995). Neural tube and notochord promote in vitro myogenesis in single somite explants. *Dev. Biol.* **167,** 87-103.

Sternberg, E. A., Spizz, G., Perry, W. M., Vizard, D., Weil, T. and Olson, E. N. (1988). Identification of upstream and intragenic regulatory elements that confer cell-type-restricted and differentiation-specific expression on the muscle creatine kinase gene. *Mol. Cell Biol.* **8,** 2896-2909.

Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J. E., deSauvage, F. and Rosenthal, A. (1996). The tumour-suppressor gene *patched* encodes a candidate receptor for sonic hedgehog. *Nature* **384,** 129-134.

Strachan, T. and Read, A. P. (1994). PAX genes. *Current Biology* **4,** 427-438.

Swalla, B. and Solursh, M. (1984). Epithelial enhancement of connective tissue differentiation in explanted somites. *J. Embryol. Exp. Morph.* **79,** 243-255.

Tajbakhsh, S. and Buckingham, M. (1994). Mouse limb muscle is determined in the absence of the earliest myogenic factor myf-5. *Proc. Natl. Acad. Sci. USA* **91,** 747-751.

Tajbakhsh, S. and Buckingham, M. (1995). Lineage restriction of the myogenic conversion factor myf-5 in the brain. *Development* **121,** 4077-4083.

Tajbakhsh, S., Vivarelli, E., Cusella-DeAngelis, G., Rocancourt, D. and Buckingham, M. (1994). A population of myogenic cells derived from the mouse neural tube. *Neuron* **13,** 813-821.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251,** 1451-1455.

Tan, S.-S., Prieto, A. L., F., N. D., Crossin, K. L. and Edelman, G. M. (1991). Cytotactin expression in somites after dorsal neural tube and neural crest ablation in chicken embryos. *Proc. Natl. Acad. Sci. USA* **88,** 6398-6402.

Tapscott, S. J., Davis, R. L., Thayer, M. J., Cheng, P. F., Weintraub, H. and Lassar, A. B. (1988). MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science* **242,** 405-411.

Tapscott, S. J., Lassar, A. B., Davis, R. L. and Weintraub, H. (1989). 5-Bromo-2-deoxyuridine blocks myogenesis by extinguishing expression of MyoD1. *Science* **245,** 532-536.

Thayer, M. J., Tapscott, S. J., Davis, R. L., Wright, W. E., Lassar, A. B. and Weintraub, H. (1989). Positive autoregulation of the myogenic determination gene MyoD1. *Cell* **58,** 241-248.

Tung, T. C., Wu, S. C., Yel, Y. F., Li, K. S. and Hsu, M. C. (1977). Cell differentiation in ascidains studied by nuclear transplantation. *Scientia Sinica* **20,** 222-233.

Vakaet, L. (1984). The initiation of gastrula ingression in the chick blastoderm. *Amer. Zool.* **24,** 555-562.

Venuti, J. M., Goldberg, L., Chakraborty, T., Olson, E. N. and Klein, W. H. (1991). A myogenic factor from sea urchin embryos capable of programming muscle differentiation in mammalian cells. *Proc. Natl. Acad. Sci. USA* **88,** 6219-6223.

Vivarelli, E. and Cossu, G. (1986). Neural control of early myogenic differentiation in cultures of mouse somites. *Dev. Biol.* **117,** 319-325.

von Kirschhofer, K., Grim, M., Christ, B. and Wachtler, F. (1994). Emergence of myogenic and endothelial cell lineages in avian embryos. *Dev. Biol.* **163,** 270-278.

Wachtler, F. and Christ, B. (1992). The basic embryology of skeletal muscle formation in vertebrates: The avian model. *Seminars in Dev Biol* **3,** 217-227.

Wachtler, F., Christ, B. and Jacob, H. J. (1981). On the determination of mesodermal tissues in the avian embryonic wing bud. *Anat. Embryol.* **161,** 283-289.

Wachtler, F., Christ, B. and Jacob, H. J. (1982). Grafting experiments on determination and migratory behaviour of presomitic, somitic and somatopleural cells in avian embryos. *Anat. Embryol.* **164,** 369-378.

Waddington, C. H. (1932). Experiments in the development of chick and duck embryos cultivated *in vitro*. *Philos. Trans. Roy. Soc. Lond. (Biol).* **13,** 221.

Wang, C. and Lehmann, R. (1991). *Nanos* is the localized posterior determinant in *Drosophila*. *Cell* **66,** 637-647.

Wang, Y., Schnegelsberg, P. N., Dausman, J. and Jaenisch, R. (1996). Functional redundancy of the muscle-specific transcription factors Myf5 and myogenin. *Nature* **379,** 823-825.

Waterson, R., Fowler, I. and Fowler, B. J. (1954). The role of the neural tube and notochord in development of the axial skeleton of the chick. *Am. J. Anat.* **95,** 337-400.

Weinberg, E. S., Allende, M. L., Kelly, C. S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O. G., Grunwald, D. J. and Riggleman, B. (1996). Developmental regulation of zebrafish MyoD in wild-type, *no tail* and *spadetail* embryos. *Development* **122,** 271-280.

Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R. and Hollenberg, S. (1991). The myoD gene family: a nodal point during specification of the muscle cell lineage. *Science* **251,** 761-766.

Weintraub, H., Tapscott, S. J., Davis, R. L., Thayer, M. J., Adam, M. A., Lassar, A. B. and Miller, A. D. (1989). Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. *Proc. Nat. Acad. Sci., USA* **86,** 5434-5438.

White, N. K., Bonner, P. H., Nelson, D. R. and Hauschka, S. D. (1975). Clonal analysis of vertebrate myogenesis. IV. Medium-dependent classification of colony-forming cells. *Dev. Biol.* **44,** 346-361.

White, N. K. and Hauschka, S. D. (1971). A new conditioned medium effect on colony differentiation. *Exp. Cell Res.* **67,** 479-482.

Whittaker, J. R. (1973). Segregation during ascidian embryogenesis of egg cytoplasmic information for tissue-specific enzyme development. *Proc. Nat. Acad. Sci. USA* **70,** 2096-2100.

Whittaker, J. R. (1982). Muscle cell lineage can change the developmental expression in epidermal lineages cells of ascidian embryos. *Dev. Biol.* **93,** 463-470.

Williams, B. A. and Ordahl, C. P. (1994). Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. *Development* **120,** 785-796.

Williams, B. A. and Ordahl, C. P. (1996). Manipulation of the avian segmental plate *in vivo*. In *Methods in Cell Biology* (ed. M. Bronner-Fraser), pp. 81-92. Academic Press, San Diego.

Woloshin, P., Song, K., Degnin, C., Killary, A. M., Goldhamer, D. J., Sassoon, D. and Thayer, M. J. (1995). MSX1 inhibits myoD expression in fibroblast x 10T1/2 cell hybrids. *Cell* **82,** 611-620.

Wolpert, L. (1971). Positional information and pattern formation. *Curr. Top. Dev. Biol.* **6,** 183-224.

Wright, W., Sassoon, D. and Lin, V. (1989). Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell* **56,** 607-618.

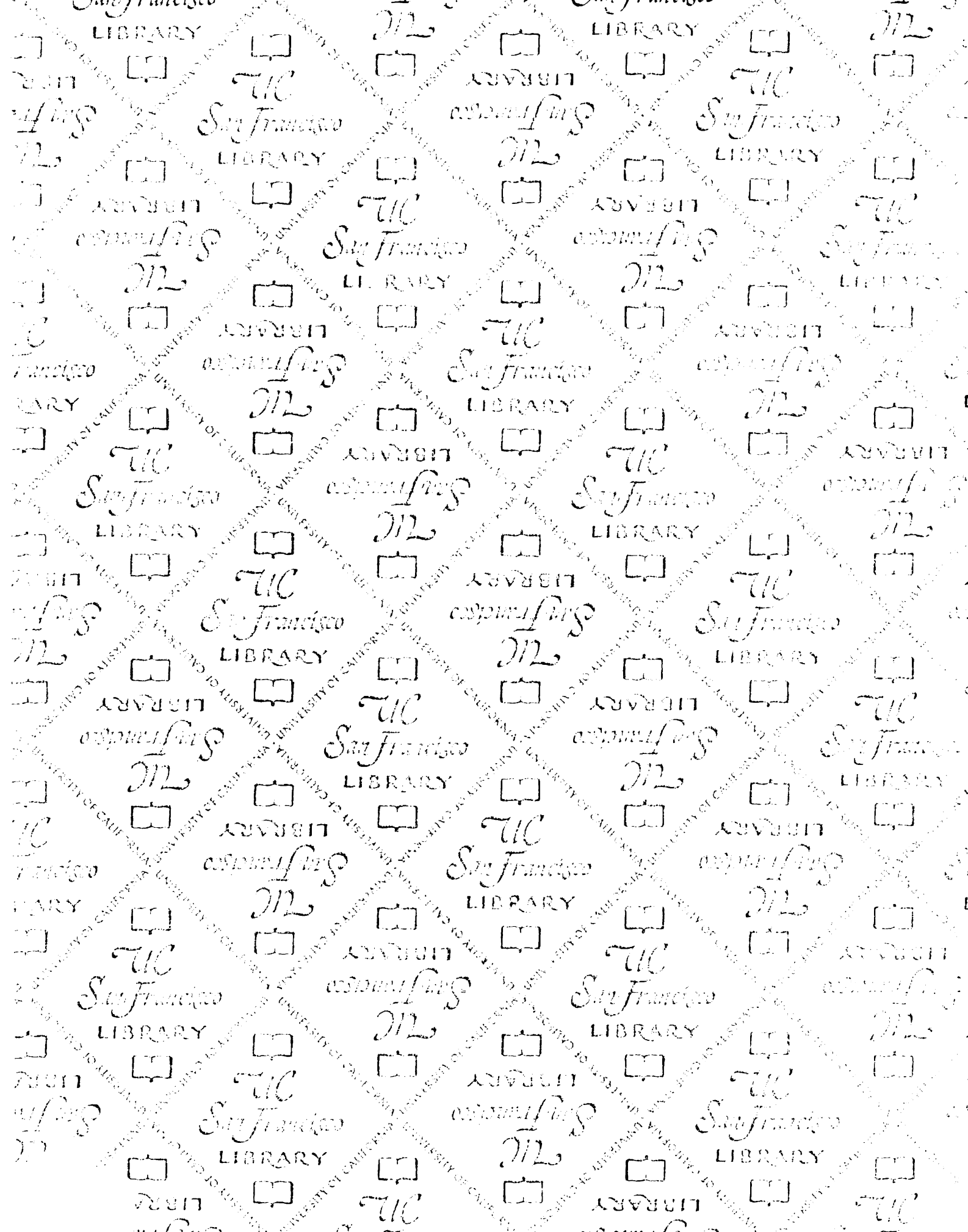
Yaffe, D. (1968). Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Natl. Acad. Sci. USA* **61,** 477-483.

Yang, Y. and Niswander, L. (1995). Interaction between the signaling molecules WNT7a and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. *Cell* **80,** 939-947.

Yee, S. P. and Rigby, P. W. (1993). The regulation of myogenin gene expression during the embryonic development of the mouse. *Genes Dev.* **7,** 1277-1289.

Yu, Y.-T., Breitbart, R. E., Smoot, L. B., Lee, Y., Mahdavi, V. and Nadal-Ginard, B. (1992). Human myocyte-specific enhancer factor 2 comprises a group of tissue-restricted MADS box transcription factors. *Genes Dev.* **6,** 1783-1798.

Zimmerman, L. B., De Jesus-Escobar, J. M. and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86,** 599-606.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