The pre-Mendelian, pre-Darwinian world: Shifting relations between genetic and epigenetic mechanisms in early multicellular evolution

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The reliable dependence of many features of contemporary organisms on changes in gene content and activity is tied to the processes of Mendelian inheritance and Darwinian evolution. With regard to morphological characters, however, Mendelian inheritance is the exception rather than the rule, and neo-Darwinian mechanisms in any case do not account for the origination (as opposed to the inherited variation) of such characters. It is proposed, therefore, that multicellular organisms passed through a pre-Mendelian, pre-Darwinian phase, whereby cells, genes and gene products constituted complex systems with context-dependent, self-organizing morphogenetic capabilities. An example is provided of a plausible 'core' mechanism for the development of the vertebrate limb that is both inherently pattern forming and morphogenetically plastic. It is suggested that most complex multicellular structures originated from such systems. The notion that genes are privileged determinants of biological characters can only be sustained by neglecting questions of evolutionary origination and the evolution of developmental mechanisms.

[Newman S A 2005 The pre-Mendelian, pre-Darwinian world: Shifting relations between genetic and epigenetic mechanisms in early multicellular evolution; *J. Biosci.* **30** 75–85]

1. Introduction

Mendelism and Darwinism are held to be the two pillars upon which modern biology is built. There are many examples in the sciences where important steps have been taken and superseded, without the discovered principles solidifying into reigning doctrines, referred to even in the breach. Experimental physicists, for example, do not continually feel compelled to explain away the deviations of their results from Newton's laws, nor do chemists incessantly note when atoms violate Dalton's precepts of indestructibility and immutability. Not so with Mendelism and Darwinism. A century and a half after Mendel and Darwin articulated their major concepts, few biologists feel comfortable reporting on agreement, or more often, deviations, from the predictions of these models without remarking on the doctrines by name. As of mid-February, the term 'Mendelian' has appeared more than 50 times in Medline listed papers for the year 2005; 'Darwinian,' despite the fact that Medline does not list the major sources in evolutionary biology, appeared in this database more than 70 separate times in 2004.

Given the dominating role of these doctrines in modern biology, it is reasonable to ask the following questions:

(i) What is the minimal statement of each of these precepts that would invite the broadest assent among biologists?
(ii) Is either principle, in its minimal form, essential to what we understand to be the nature of living organisms?
(iii) Insofar as either principle is true about modern organisms, has it always been true in the same fashion throughout evolutionary history?

I will attempt to answer each of these questions in what follows. There are well-known deviations from both Mendelism and Darwinism, but they are usually consid-

Keywords. Baldwin effect; EvoDevo; limb development; plasticity

ered the kind of exceptions that 'prove the rule.' Since my ultimate conclusion is that the answers to the second and third questions are both 'No,' I will attempt to show how the exceptions, rather than proving that the rules are valid, indicate instead the fluid, evolutionarily-changing character of biological organization. In particular, I will suggest that not only have phenotypes and genotypes changed over the course of evolution, but that the manner in which the genotype exerts its influence over the phenotype (which is the common purview of Mendelism and Darwinism) has also changed (Newman and Müller 2000; Newman 2003).

Organismal characters that exhibit Mendelian patterns of inheritance and are subject to Darwinian modes of evolution can be analysed in a useful fashion through the lens of the genes involved in generating these features. This has led in many cases to genetic reductionist and determinist styles of explanation, where the genes are ascribed excessive causal agency. One purpose of this article is to argue that while genes and their products have always been essential components of biological systems, it is only after organisms evolved their way into the Mendelian/Darwinian world that their characters could (plausibly, but I suggest, incorrectly) be considered primarily to be expressions of their genes. I will confine my discussion to morphological features of the metazoa, i.e. multicellular animals; similar analysis could be done at other levels of biological organization. I will end with a concrete example of metazoan organ formation – the vertebrate limb – and use it to demonstrate the conclusion that both the Mendelian and Darwinian character of contemporary living system are products of evolution, not essential to organismal existence.

2. Mendelism: rules and exceptions

Gregor Mendel (1822–1884) was not an evolutionist. As a 19th century Augustinian Catholic monk, and later abbot of his monastery, he was, in fact, officially a proponent of special creation. Like most scientific thinkers of the time (a community to which he belonged, notwithstanding the myth of his intellectual isolation; Orel 1996), Mendel believed that new species could form by hybridization. This type of innovation, which indeed occurs in plants, was not considered to violate the Biblical accounts of creation (Callender 1988). Mendel's work with peas and with hawkweeds considered both the inheritance of alternative versions ('traits') of the same character (i.e. flower colour, seed shape or texture) within a given species, as well as the long-term stability of hybrids formed between pre-existing species. However, the 'factors' or 'elements' that he adduced to account for the mathematical regularity of his results with peas (the only actual law that Mendel ever enunciated he called the 'Law Valid for *Pisum*;' Stern and Sherwood 1966), though they existed in distinct versions, were held to be immutable.

A boulder in a riverbed can divert the stream in one direction or another without being causally involved in the production of the flowing water. Similarly, in biological systems with the potential to exhibit alternative versions of the same character, the switching element may have very little to do with how the character itself is constructed. Nonetheless, some of Mendel's later adherents assumed his elements to be causal determinants of the characters with which they were associated. The botanist Hugo de Vries (1848–1935), for example, stated in 1889: "If one considers the species characters in the light of the doctrine of descent, it then quickly appears that they are composed of separate more or less independent factors" (quoted in Dunn 1965, p. 41). He saw no reason to change his opinion in 1900 after he became aware of Mendel's work. The evolutionary biologist and historian Ernst Mayr (1904–2005) referred to such notions as 'beanbag genetics,' but nonetheless asserted that Mendel's major contribution to biology was "[h]is inference that each character is represented in the fertilized egg by two, but only two, factors, one derived from the father and the other from the mother, and that these could be different" (Mayr 1982).

During the early part of the 20th century Mendelian factors came to be identified with genes, sequences of DNA that specify proteins or RNA molecules. Allelic variations in corresponding DNA sequences in chromosome pairs are indeed transmitted from generation to generation according to Mendelian principles. But Mayr's formulation suggests that characters also (typically) follow these rules. Since characters are rarely the direct manifestation of a single gene or gene product, however, the fact that genes are inevitably associated with any traits does not carry any implication about the trait's being inherited in a Mendelian fashion. According to the Danish botanist Wilhelm Ludwig Johannsen (1857–1927), writing soon after Mendel's work entered the scientific mainstream, "By no means…have we any right to conceive that each special gene (or a special kind of gene) corresponds to a particular phenotypic unit-character or (as morphologists like to say) a 'trait' of the developed organism" (quoted in Dunn 1965, p. 93). The British geneticist William Bateson (1861–1926) similarly rejected the notion that evidence that a character is influenced by a Mendelian-type factor implies that the factor itself 'represents' the character (Bateson 1913). Like Mendel, he saw the factors as 'differentiating elements' operating within what we would now refer to as complex dynamical systems (Newman 2005).

While classical Mendelian factors are either dominant or recessive, there are many instances of incomplete dominance, where the phenotype is intermediate between

that associated with either allele alone. Mendel himself found this in snapdragons, which is why his law was stipulated as 'valid for peas.' Human blood groups, among other characters, exhibit codominance, where two different alleles are both expressed and yield a phenotype qualitatively different from that associated with either allele. In polygenic inheritance (the most common situation) two or more factors enter into the production of a character. Here patterns of inheritance are often complex because of nontransitive dominance relationships, and may indeed be skewed because of alleles that are lethal in the homozygous state, as is the case for the yellow coat colour in mice (Michaud *et al* 1993).

Inheritance can also be multifactorial, where inherited factors interact with determinants of the gestational or external environment. A classic example of this is the formation of cervical vertebrae in mice, where a different strain's gestational environment can in certain cases override the 'genetically-determined' number of bones (McLaren and Michie 1958). Genes, moreover, are subject to imprinting, where activity of one or another allele is suppressed by a potentially reversible chemical modification, such as DNA methylation. This can be influenced by the external environment. For example, methylation, and thus efficiency in driving transcription, of the promoter region of the glucocorticoid receptor gene in rat pups, is influenced in a persisting fashion by various maternal grooming and nursing behaviours (Weaver *et al* 2004). Paramutation is a stable, heritable change in transcription of one allele caused by interaction with the other (Hollick *et al* 1997), a phenomenon that can be mediated by the physical arrangement of genes within chromosomes (Reilly 2004) and which undermines the separability of Mendelian factors.

Although imprinting and paramutation confound Mendelian patterns of inheritance, they exist within a 'Mendelian' framework in which each factor is present in two allelic forms, that is, the framework alluded to by Mayr in his statement, quoted above, that Mendel's main finding was that "each character is represented in the fertilized egg by two, but only two, factors." But in addition to the various complications just described that lead to non-Mendelian inheritance in diploid organisms, it must be recognized that not every organism, nor even every multicellular, morphologically differentiated organism, is diploid (cf. the cellular slime mould, *Dictyostelium*). Finally, regardless of whether an organism is haploid or diploid, a large proportion of its genes will participate in several biological processes. This pleiotropy makes futile nearly every attempt to associate individual genes with individual characters.

The modern concept of the gene (in its plurality of meanings, see Griffiths and Neumann-Held 1999; Moss 2003) is obviously a lineal descendent of Mendel's factor, but it is far from the same thing. While many traits, diseases, etc. are inherited in a Mendelian fashion with respect to one or another gene, the notion that characters are represented (in any sense of this word) by the genes that influence their expression is incorrect. It only to be expected that a century after Mendel's breeding experiments entered the scientific mainstream the concepts that he and his direct successors used to account for his results have undergone revision. What is unusual, as noted at the beginning of this essay, is that Mendelian inheritance, with the tacit implication that there are factors that 'correspond' to characters or traits, is conceptually still the default option, with exceptions to the option having to be noted. This situation is simultaneously a source and a product of the persistent ideology of genetic determinism.

Considering all the described limitations and qualifications, a minimal definition of the 'Mendelian' quality of organisms, which is subject to the fewest exceptions and which would have been recognizable to Mendel himself, would perhaps be that (i) all organisms contain numerous genes which they pass on to their descendents; (ii) each gene can exist in multiple versions; and (iii) there is a predictable association between different versions of a specific gene and different versions of a corresponding variable character. But even this minimal characterization of Mendelism has many exceptions in multicellular organisms, mainly relating to point (iii). Was Mendelian inheritance, then, some primitive, defining state of organisms that has been complicated and obscured by subsequent evolution? The evidence suggests that precisely the opposite is true: in those rare cases where unique relationships exist between a specific character and one or another version of a specific gene, i.e. the character is Mendelian in the strict sense defined by Mayr or in the relaxed sense described above, it is reasonable to infer that the condition was achieved over the course of evolution rather than having been present from the start (Newman and Müller 2000). If this is the case, the ensemble of the primitive metazoans, was indeed a 'pre-Mendelian' world of organisms.

3. Darwinism: origins, meaning, limitations

The recognition that life on earth has an evolutionary history, indications of which can be found in the Brihadaranyaka Upanishad (ca. 600 BC) and the writings of the pre-Socratic Greek philosopher Empedocles (ca. 450 BC), was introduced into modern scientific discourse by Jean-Baptiste Lamarck (1744–1829) in the early 19th century (Lamarck 1809), and later provided with the plausible mechanism of natural selection by Charles Darwin (1809–1882) (Darwin 1859). Darwin's theory did not depend on any particular concept of the gene, though he

had his own theory of 'pangenesis,' involving 'gemmules,' factors directly tied to the identity of the various organs which flowed through the body and were carried to the next generation by the germ cells. While Darwin's pangenesis theory is now dismissed, the implied notion that all features of an organism subject to heritable variation are built from a finite set of discrete factors, in other words, that inheritance is 'particulate,' was an important component of the mid-20th century neo-Darwinian synthesis.

As we have seen above, the developing understanding of the role of genes provided no warrant for this picture: while inheritance of genes themselves can be said to be particulate, inheritance of characters is certainly not. But whereas the neo-Darwinian synthesis thus echoed an incorrect aspect of Darwin's views, it strenuously denied another aspect of Darwin's thinking which became increasingly prominent in the later editions of the "*Origin of Species*." This is the notion of 'soft' inheritance, whereby the efficacy of transmission of characters was subject to use and disuse and other conditional effects. The Lamarckian tenet of inheritance of acquired characteristics, now rejected by Darwinians and most critics of Darwinism alike, was, ironically, a view to which Darwin himself had become more sympathetic as he modified his theory (Darwin 1872) in response to arguments of his critics, such as Fleeming Jenkin, on the limits of natural variation and selection in their capability to effect macroevolution.

The problem in its starkest form is the lack of any evidence from animal breeding or field observations that incremental Darwinian mechanisms can do anything but modify and elaborate on pre-existing structural motifs. As Jenkin stated of Darwin's theory, in a formulation that a century and a half later still does not find an answer within the strict framework of neo-Darwinism: "That theory rests on the assumption that natural selection can do slowly what man's selection does quickly; it is by showing how much man can do, that Darwin hopes to prove how much can be done without him. But if man's selection cannot double, treble, quadruple, centuple, any special divergence from a parent stock, why should we imagine that natural selection should have that power?" (Jenkin 1867).

On the other hand, Lamarckian mechanisms, if they existed, could move things forward. Assume that organisms have inherent morphological plasticity that can be elicited by external influences in a consistent fashion in multiple members of the same population. If the propensity to assume one or the other of these 'ecophenotypes' could be imprinted on the germ plasm by the different conditions of existence, phenotypic divergences from the parental stock would be accelerated. As Darwin recognized, this potential mode of organismic change could complement natural selection.

Darwin's embrace of a Lamarckian position represented limitations in the explanatory power of his theory which nonetheless could not be overcome by this maneuver: the preponderance of evidence in the period since Darwin wrote has taught us that acquired characteristics are, in general, not inherited. Furthermore, inherited alterations in traits are typically associated with gene variations, apparently supporting 'hard' over 'soft' inheritance. The population geneticist R A Fisher, whose quantitative analyses are a cornerstone of the neo-Darwinian synthesis, argued persuasively that 'genes of large effect,' i.e. allelic variants, the expression of which induce major phenotypic change in individual organisms, are unlikely to be established in a population at sufficiently high frequency to be a major mode of evolutionary change (Fisher 1930). This essentially squelched consideration of the 'hopeful monster' scenario for macroevolution proposed by the developmental geneticist Richard Goldschmidt (Goldschmidt 1940). The full story has not yet been written on this, however. Despite Fisher's arguments, genes of large effect apparently are present in *Drosophila* (Tautz 1996). Moreover, in plants, which frequently provide exceptions to zoocentric rules, genes of large effect not only exist, but play demonstrated roles in speciation (Gottlieb 1984; Bradshaw and Schemske 2003).

These findings are difficult to incorporate into standard Darwinian accounts of evolution, and may indeed represent rare exceptions. A minimal statement of modern Darwinism, therefore, in analogy to the one formulated above for modern Mendelism, would have the following elements: (i) organisms in a population vary phenotypically; (ii) phenotypic variations are subject to selection and only those associated with genotypic variations leave an imprint on the evolutionary record; (iii) evolutionary change is a gradual process.

In the case of Mendelism, we saw that even in its minimal characterization it only pertains to exceptional cases. With respect to neo-Darwinism, we may acknowledge that such mechanisms do pertain generally to modern organisms, particularly with respect to continuous variation, but question (because of issues similar to those that persuaded Darwin to modify his views) whether these mechanisms are capable of producing significant morphological innovations (Müller and Wagner 2003) and large-scale, macroevolutionary change. Neo-Darwinism contends that macroevolution is just the result of long-term accumulation of microevolutionary changes. But there is little direct evidence for this; it seems to be a matter of faith in the absence of other possibilities. Fortunately there are other possibilities: properties of contemporary organisms ignored by neo-Darwinism provide a window into likely mechanisms of large-scale morphological and other phenotypic changes earlier in the history of multicellular life. For reasons similar to those discussed in the

previous section, these organismal tendencies would have been suppressed over the course of evolution.

Here I will briefly mention some of these evolution-promoting properties; they are discussed in detail in the references cited.

(i) Organisms exhibit a great deal of phenotypic plasticity. This is a commonplace in plants, which can have widely different morphotypes if grown under different conditions. It is true about the cellular slime moulds such as *Dictyostelium discoideum*, the stages of whose 'life cycles' can also be viewed as distinct environment-dependent morphological phenotypes (Bonner 1967). Although not generally recognized, morphological plasticity is also seen in vertebrates. The strain-associated number of cervical vertebrae in mice, mentioned above as an example of multifactorial inheritance, is also an example of phenotypic plasticity, since vertebrae number can be changed by transferring embryos to the uterine environment of a strain with a different characteristic number (McLaren and Michie 1958). West-Eberhard (2003) provides many other examples of phenotypic plasticity across taxonomic groups. Although it could be argued that some cases of environmentally-dependent plasticity represent distinct developmental pathways that have evolved to generate different functional outcomes (temperature-dependent sexual determination in alligators, for example), in other cases, like that of cervical vertebrae development, it most likely represents inherent condition-dependent variability in the generative system, with no functional implication. Computational modelling has shown that, under circumstances in which its extent has a variable genetic aspect, plasticity can actually speed up evolution (Behera and Nanjundiah 2004).

(ii) The environment in many cases is not simply a 'trigger' of morphological diversification, but is part and parcel of the developmental system. Gilbert (2005) gives several examples, among which are the dependency of intestinal development in mammals and the development of gut-associated lymphoid tissue on different populations of resident microbes. Here it must be recognized that changes in the external environment may be responsible not only for choices between outcomes inherent to the biological system, but may change the nature of the system, leading to entirely unprecedented features.

(iii) Changes in organismal phenotype resulting from elicitation of plastic responses can be transmitted across generational lines by 'epigenetic' means, referring in the most general sense to all enduring alterations that do not involve changes in gene sequence. Trivially, if an environmental change (e.g. a change in ambient temperature or salinity) persists, the new plasticity-dependent phenotype will persist. Examples, including many relating to behaviour, are discussed by Johnston and Gottlieb (1990) and Oyama (2000). But 'epigenetic' is also used in a more narrow sense to refer to chemical alterations in genes (e.g. methylation of cytosine, a mechanism of imprinting, described above) and other persistent but potentially reversible changes in gene expression that control gene expression and phenotypic variability. Such epigenetic changes do not depend on environmental constancy; in this sense they are portable. But neither are they permanent changes in the genotype. In the example mentioned above of potentially reversible changes in methylation of the glucocorticoid receptor gene promoter in rat pups tied to different maternal behaviours, the changes persisted into adulthood and affected, in turn, the behaviour of the offspring (Weaver *et al* 2004). Jablonka and Lamb (1995) suggest that such epigenetic changes can be the starting point of new evolutionary trajectories. Drift or selection may lead to genetic change that consolidates or reinforces the epigenetically-acquired phenotype (mutations in *cis*-regulatory sequences of developmentally-involved genes, like those described by Dayal *et al* 2004, may accomplish this), but here the genetic change follows, rather than precedes, the evolutionary step. Domestication, the paradigmatic case in Darwin's theory for evolution in action, appears to depend (at least in the best studied case; Trut 1999) on a scenario that initially involves this type of epigenetic change.

(iv) Even in those cases where plasticity-based phenotypic alteration is not stabilized by an epigenetic change (i.e. where it is solely dependent on environmental influences), it can still provide a bias in the developmental system which can be stabilized by random gene changes. This scenario of "phenotype first, genotype later," especially with regard to the role of behaviour in evolution, is referred to as the Baldwin effect (Baldwin 1902), and has been discussed more recently as an evolutionary mechanism by Patrick Bateson (2005) and West-Eberhard (1998, 2003). We have generalized this notion to include the effects on evolution of physical and other epigenetic mechanisms of morphogenesis (Newman and Müller 2000; see also Müller 1990 and Newman and Comper 1990). West-Eberhard writes of "genetic accommodation – adjustment of the frequency of occurrence of a phenotypic trait due to selection on genetic variation in the polygenic regulatory mechanisms influencing its threshold of expression" (West-Eberhard 1998). The biological plausibility of epigenetically-driven evolutionary scenarios is supported by computational models (Pal and Miklos 1999; Salazar-Ciudad *et al* 2001). Indeed, the neo-Darwinian paradigm has a place for this sort of mechanism, but it is a minor one (Simpson 1953).

We can now ask a question about the incremental, neo-Darwinian mode of evolution similar to the one we asked previously about Mendelian inheritance: has it been the

primary means of transformation of biological forms throughout the history of multicellular organisms? Again, we have suggested that the exact opposite is the case: organisms that existed before extensive stabilizing evolution (Schmalhausen 1949) consolidated the relationship between particular phenotypes and particular genotypes were more susceptible to Baldwinian evolution than are modern-day organisms (Newman 1994; Newman and Müller 2000). Over time, as genetic mechanisms evolved to make organisms' developmental trajectories more reliable (what Waddington referred to as 'canalization;' Waddington 1942) and their physiological states more homeostatic, phenotypic plasticity would have been suppressed. Put another way, the evolutionary forces that turned organisms into Mendelian and Darwinian entities simultaneously marginalized the Baldwin effect, with its capacity to mobilize and assimilate genetically, large-scale changes in phenotype, as a mode of evolution. This, then, is a way out of the conundrum that caused Darwin, incorrectly, to move toward a Lamarckian stance. Modern-day organisms cannot exploit large-scale phenotypic changes based on inherent plasticity to make evolutionary leaps. In contrast, their developmentally less-constrained ancestors are likely to have done so. Macroevolution (e.g. the formation of new body plans, or major innovations like the vertebrate limbs), in this analysis, is a thing of the past.

4. Developmental plasticity in the vertebrate limb and a conjecture on limb evolution

In the scenario outlined in the preceding sections, while there is an overall tendency for organisms to evolve away from the pre-Mendelian, pre-Darwinian world, they would not do so in a uniform fashion; indeed individual modern-day species exhibit Mendelian or Darwinian properties (as defined above) to different extents in different organs and subsystems. To make the above observations more concrete, I will now describe what we believe to be the core mechanism underlying the development of the vertebrate limb skeleton (Hentschel *et al* 2004). In modern-day vertebrate animals this structure clearly has some variants that are subject to Mendelian inheritance patterns (Wilson 1998) and has equally clearly been subject to morphological fine-tuning by incremental Darwinian mechanisms over the course of recent evolution (Shapiro *et al* 2003). Our proposed core mechanism employs only a tiny proportion of the dozens of genes found to be involved in limb skeletal pattern formation (Tickle 2003) and achieves patterning in an 'emergent' (Salazar-Ciudad *et al* 2001) fashion, making use of self-organizing properties of biochemical and cellular systems (Turing 1952; Meinhardt and Gierer 2000; Miura and Maini 2004; Newman and Forgacs 2005). Systems of this sort are highly versatile in their capacity to produce patterns, but the patterns are also subject to profound alterations as a result of changes in parameters such as enzymatic turnover rates and tissue growth rates and, in some cases, initial conditions, such as the concentration of a key factor at the time pattern formation begins. These are things that can readily be brought under control by adding, tuning, and integrating gene-gene relationships during the course of evolution. Our core, or 'bare bones' (Hentschel *et al* 2004), mechanism for limb skeletogenesis (or something very similar to it) is therefore hypothesized to have been the originating mechanism for this process at the pre-Mendelian, pre-Darwinian stage of limb evolution.

The limbs form from mounds of tissue ('limb buds'), which emerge from the body wall, or flank, at four discrete sites – two for the forelimbs and two for the hindlimbs. The mesenchymal tissue of the early limb bud, which gives rise to the skeleton and muscles, forms a paddle-shaped tissue mass referred to as a 'mesoblast,' surrounded by a layer of simple epithelium, the ectoderm. The skeletons of all vertebrate limbs develop in a proximodistal fashion: that is, the structures closest ('proximal') to the body form first, followed, successively, by structures more and more distant ('distal') from the body. For the forelimb of the chicken, for example, this means the humerus of the upper arm is generated first, followed by the radius and ulna of the mid-arm, the wrist bones, and finally the digits (figure 1).

The bones of the limb skeleton do not arise directly as bone tissue. The pattern is first laid out as cartilage, which is replaced by bone later during embryogenesis in most, but not all vertebrate species. Some salamanders, for example, have limb skeletons composed largely of cartilage.

Before the cartilages of the limb skeleton form, the interior, loosely packed (mesenchymal) cells of the mesoblast are dispersed in a hydrated extracellular matrix (ECM). The first morphological evidence that cartilage will differentiate at a particular site in the mesoblast is the emergence of precartilage mesenchymal condensations: transient aggregations of cells within a mesenchymal tissue. The cells at these sites then progress to fully differentiated cartilage elements by switching their transcriptional capabilities. Condensation is mediated first by the local production and secretion of ECM glycoproteins, such as fibronectin (Tomasek *et al* 1982; Kosher *et al* 1982), which acts to trap the cells in specific places (Frenz *et al* 1989a,b). The aggregates are then consolidated by direct cell-cell adhesion (Oberlender and Tuan 1994) before they differentiate into cartilage and move apart once again (Newman and Tomasek 1996).

Because all the precartilage cells of the limb mesoblast are capable of producing fibronectin and condensing, but

only those at sites destined to form skeletal elements actually do so, there clearly must be communication among the cells to divide the labor. This communication is mediated in part by secreted, diffusible factors of the TGF-*b* family of growth factors, which promote the production of fibronectin (Leonard *et al* 1991). Limb bud mesenchyme also shares with many other connective tissues the capability of producing more TGF-*b* upon stimulation with this factor (Miura and Shiota 2000). That is, TGF-*b* is positively autoregulatory in limb bud mesenchyme.

The limb bud surface tissue, the ectoderm, performs several important functions. First, it is a source of fibroblast growth factors (FGFs) (Martin 1998). Although the entire limb ectoderm produces FGFs, the particular mixture produced by the apical ectodermal ridge (AER), a narrow band of specialized ectodermal cells running in the anteroposterior direction along the tip of the growing limb bud in birds and mammals, is essential to limb outgrowth and pattern formation. The AER keeps the precondensed mesenchyme of the 'apical zone' in a labile state (Kosher *et al* 1979) and its removal leads to terminal truncations of the skeleton (Saunders 1948).

The FGFs produced by the ectoderm affect the developing limb tissues through one of three distinct FGF receptors. The apical zone is the only region of the mesoblast-containing cells that express FGF receptor 1 (FGFR1) (Peters *et al* 1992; Szebenyi *et al* 1995). In the developing chicken limb, cells begin to condense at a distance of approximately 0·3 mm from the AER. In this 'active zone' FGFR1 is downregulated and cells that express FGFR2 appear at the sites of incipient condensation (Peters *et al* 1992; Szebenyi *et al* 1995; Moftah *et al* 2002). Activation of these FGFR2-expressing cells by FGFs releases a laterally-acting (that is, peripheral to the condensations) inhibitor of cartilage differentiation (Moftah *et al* 2002). Finally, differentiated cartilage in the more mature region, proximal to the condensing cells, expresses FGFR3, which is involved in the growth control of this tissue (Ornitz and Marie 2002). We refer to the region containing mature cartilage as the 'frozen zone.'

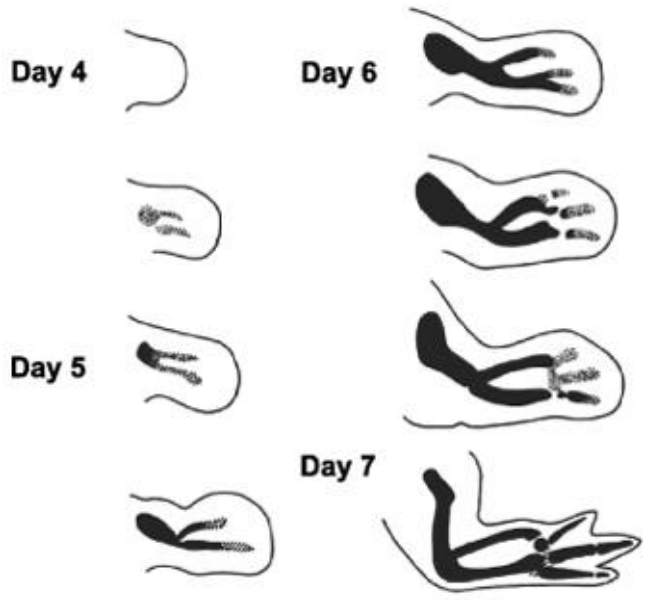

Figure 1. Progress of chondrogenesis in the chick wing bud between 4 and 7 days of development, shown in cross-section. The stipple represents precartilage; the solid black definitive cartilage. For each figure, proximal is to the left, distal to the right, anterior up and posterior down. The dorsal to ventral direction (dorsoventral axis) is perpendicular to the plane of the picture. Adapted, with changes, from Newman and Frisch (1979).

A model that incorporates all the above ingredients (presented in schematic form in figure 2) must involve a complicated set of mathematical equations representing the influences of the various mentioned genes on one another via their products, as well as the diffusion of released signal molecules ('morphogens') such as TGF-*b* and FGF through the ECM and growth of the different tissue domains. We devised a set of eight ('coupled nonlinear partial differential') equations that encompass the interactions in figure 2 (Hentschel *et al* 2004). It should be recognized that the limb bud here is represented as a two-rather than three-dimensional structure (the thickness of the limb from back to front is collapsed to zero), with a rectangular, rather than curvilinear contour. Moreover, cell density is represented as a continuous variable rather than a collection of discrete entities that may become packed to different extents. Using mathematical techniques, we confirmed that nonuniform patterns of cell density indeed arise from this system (Alber *et al* 2005).

As simplified as this system is relative to the interactions in the actual developing limb, computer simulation of a set of equations of this complexity is not feasible. We therefore applied some reasonable biologically-motivated assumptions using estimates of timescales of various processes, reducing the eight equations to four. Then, using mathematical simplifications based on expectations concerning the behaviour of functions involved we were able to simulate the system under realistic growth dynamics for the various domains (Hentschel *et al* 2004). As seen in figure 3, the pattern of 'bones' that this system predicts is decidedly limb-like (given the constraints noted above). It is significant that the system exhibits somewhat different patterns when different initial conditions are used. A further reduction in complexity of the system of Hentschel *et al* (2004) to two equations describing simply the interaction and diffusion of the chemical activator and inhibitor of condensation, permitted it to be embedded in a more realistic computational frame-

work in which cells were represented as extended, discrete objects and the limb bud was rendered three-dimensionally. These simulations also yield limb-like skeletal patterns (Chaturvedi *et al* 2005). Interestingly, small variations of parameters (rate constants, diffusion coefficients) in each of these computational models of the skeletal pattern forming mechanism led to large changes in morphology – polydactyly, fused elements, etc. In a sense, all the genes in this core mechanism are genes of large effect.

From such overlapping tests of the hypothesized core mechanism, each with its different approximations and limitations, we can be fairly confident that the features of the developing limb captured by our model constitutes a mechanistic basis for skeletal patterning. Whether it was the actual originating mechanism by which limbs first appeared in vertebrate ancestors approximately 400 million years ago is impossible to ascertain with currently available information. What is clear, though, is that if the originating mechanism was anything like that discussed here (a reasonable supposition), its immediate response to gene mutation, or to environmental perturbation during development such as changes in ambient temperature, would be neither Mendelian nor Darwinian. Either type of alteration could yield large-scale changes in pattern: missing elements, fused elements, seven digits rather than three or five, and so on. It is equally clear, however, that skeletal patterns produced by such mechanisms could have served as templates for the accumulation, by stabilizing natural selection (Schmalhausen 1949), of

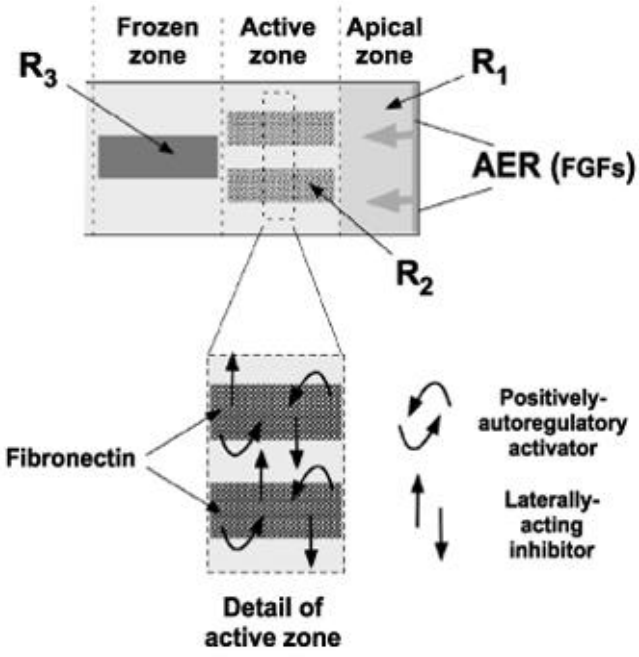

Figure 2. Schematic representation of the biochemical-genetic circuitry underlying the pattern forming instability described in the model of Hentschel *et al* (2004), superimposed on a two-dimensional representation of the 5 day limb bud shown in figure 1. Positive autoregulation of TGF-*b*, induction of fibronectin by TGF-*b*, promotion of precartilage condensation by fibronectin, and FGF-dependent elicitation of a lateral inhibitor of cartilage development from sites of condensation, are all supported by experimental evidence. The model assumes the inhibitor acts directly on TGF-*b*. In the apical zone cell rearrangement is suppressed by the FGFs emanating from the AER. The active zone is the site of spatiotemporal regulation of mesenchymal cell condensation (i.e. pattern formation). When cells leave the proximal end of the active zone and enter the frozen zone they differentiate into cartilage and their spatiotemporal pattern becomes fixed. Proximal is to the left, distal to the right, anterior is upward, posterior downward. The length of the dorsoventral axis is collapsed to zero in this simplified model.

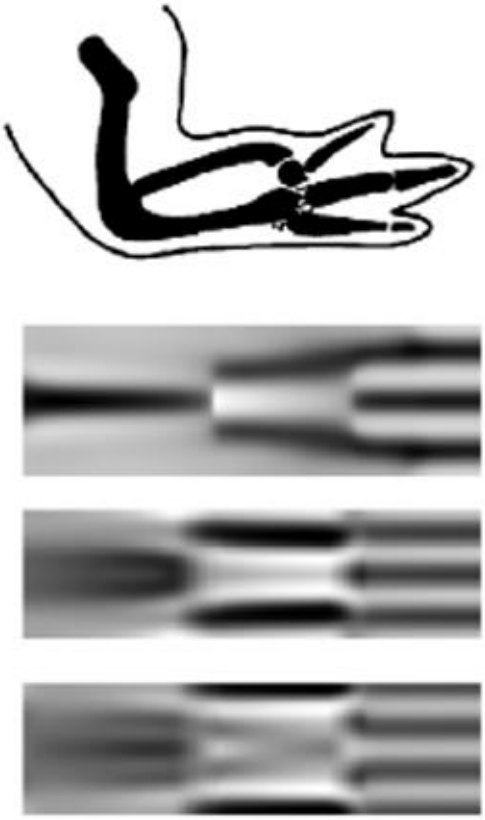

Figure 3. Simulations of limb skeletal development in the model of Hentschel *et al* (2004). Typical examples of skeletal structures generated by the model, using different initial conditions are shown in comparison to the longitudinal section of the skeleton of the chicken limb at 7 days of development shown in figure 1. The distribution of cartilage is shown in a continuous grayscale in the simulation panels, with black representing highest cartilage density. Skeletal form in the model is dependent on parameter values and time-dependent changes in the active zone (which were the same for all three simulations shown) and initial conditions (which differed, yielding slightly different patterns). See Hentschel *et al* (2004) for details.

reinforcing, developmentally canalizing genetic circuitry. After long periods of evolution, the generative mechanism would become increasingly buffered from environmental and much genetic (i.e. mutational, transcriptional) perturbation. But while many gene changes would at this point have small, or even no effect on the phenotype, in certain cases an allelic variation could control the choice between two well-buffered alternatives. Such stereotypical genotype-phenotype relationships would be identified as Mendelian inheritance patterns. With extensive stabilization of the generation of the phenotype, moreover, the only way the limb skeleton could become structurally remodeled in the course of further evolution would be by the Darwinian mechanism of gradual populational changes in the relative abundance of alleles of small effect.

5. Conclusion

The example of the limb development model shows, in principle, that biological mechanisms employing standard ingredients: cells, their genes, and the products of those genes, can produce recognizable structures that nonetheless may not abide by the principles of Mendelism or Darwinism (no matter how minimally defined) for a long time following their inception. Evolution fosters the accumulation of redundant and parallel mechanisms to ensure reliability of developmental outcome. It is therefore reasonable to suppose that the further back in evolution one goes, the greater the proportion of metazoan structures, including the basic body plans, which would have been generated by mechanisms with plastic, variable outcomes. For the presumed organisms in this pre-Mendelian, pre-Darwinian world, the dynamical interactions of genes and their products could never be confused with 'developmental programs.' Correspondingly, the morphological phenotypes generated would depend too much on physical and other system properties for genes to be considered their privileged determinants. Moreover, gene variations in this less-evolved world would be expected to have had effects that were far more context-dependent than gene variations in modern-day organisms. For organisms in our modern Mendelian, Darwinian, world, evolved relationships between phenotypes and genotypes, however real, tell us little about how they originated and took hold. Genetic determinism may sometimes work, not because it captures the essential nature of living systems, but because it ignores it.

Acknowledgments

I benefited from discussions with Gerd B Müller and Vidyanand Nanjundiah while developing the ideas in this paper. Gerd Müller and two anonymous reviewers also provided helpful criticisms of earlier drafts. Our empirical and theoretical work on vertebrate limb development has been supported by grants from the National Science Foundation Biocomplexity Initiative and Division of Integrative and Organismal Biology. My travel to the Discussion meeting on Genetic Determinism, where the talk on which this paper is based was presented, was supported by the NSF Division of International Programs.

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*e*Publication: 11 March 2005