Is Segmentation Generic?

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Summary

When two populations of cells within a tissue mass differ from one another in magnitude or type of intercellular adhesions, a boundary can form within the tissue, across which cells will fail to mix. This phenomenon may occur regardless of the identity of the molecules that mediate cell adhesion. If, in addition, a choice between the two adhesive states is regulated by a molecule the concentration of which is periodic in space, or in time, then alternating bands of non-mixing tissue, or segments, can form. But temporal or spatial periodicities in concentration will tend to arise for any molecule that is positively autoregulatory. It is therefore proposed that segmentation is a 'generic' property of metazoan organisms, and that metamerism would be expected to have emerged numerous times during evolution. A simple model of segmentation, based solely on differential adhesion and periodic regulation of adhesion, can account for segment properties as disparate as those seen in long and short germ band insects, and for diverse experimental results on boundary regeneration in the chick hind brain and the insect cuticle. It is suggested that the complex, multicomponent segment-forming systems found in contemporary organisms (e.g., *Drosophila)* are the products of evolutionary recruitment of molecular cues such as homeobox gene products, that increase the reliability and stability of metameric patterns originally templated by generic self-organizing properties of tissues.

Introduction

During the development of many animal species, tissue primordia are demarcated into a linear arrangement of structurally similar domains. This is seen, for example, in the establishment of body segments in insects such as *Drosophila*[1] and the grasshopper[2], of the blocks of bone- and muscle-forming mesoderm, termed somites, along the embryonic axis of vertebrates[3], and of the periodic swellings in the developing vertebrate hindbrain, termed rhombomeres[4,5]. The development of digits in the embryonic vertebrate limb exemplifies this process of tissue subdivision in two dimensions: first the fingers or toes are apportioned from the distal mesenchyme of the developing limb bud as a sequence of parallel lozenge-shaped cell condensations, and shortly thereafter the individual digital primordia further segment as they differentiate into chains of cartilage rods[6,7].

The evolutionary origins of segmental organization of body plans and organs have been the subject of much speculation. Willmer[8] for example, lists several 'functions and advantages' of metamerism, including facilitation of undulatory swimming or burrowing movements resulting from phased activity of a segmented nervous system, the mechanical benefits and versatility of periodic strengthening of the cuticle in arthropods, the energetic efficiencies of localized muscle contraction in annelids and chordates, and the production of larger body sizes with an economical use of genetic information. Rhombomeres have similarly been proposed to serve a function in the developmental organization of the vertebrate brain, although it is recognized that the segmental aspects of this organization are not evident in the mature organism[4].

These discussions of segmentation follow the usual neo-Darwinian analysis of organismal morphologies in assuming that the organization of body plans and organs has evolved by incremental improvements in adaptation to fixed or changing environments. This 'gradualist' view would seem to imply that the evolution of segmentation in any lineage should occur segment by segment, with each intermediate morphology undergoing the rigors of natural selection. The well-accepted idea that segmentation has arisen several times during the course of evolution[8,9] would be explained, in this view, by 'convergence' of forms as the result of similar phylogenetic histories, or by retention, in disparate lineages, of common primordial molecular mechanisms[10].

But an alternative hypothesis is that the capacity to undergo segmentation may be 'generic' to tissues, in the sense of being an outcome of their most general physical and chemical properties. A generic property is defined as one that is intrinsic to a kind of material, regardless of possible variations in its molecular makeup, such as the ability of liquids to flow, or strings to vibrate[11]. If segmentation is indeed a generic property of tissues, it would be *expected* to have arisen numerous times during evolution, be present in many phylogenetic lineages regardless of their genealogical relationships, and potentially be underlain by different molecular mechanisms in different taxa[12]. Such an intrinsic capability would also provide a basis for the 'global' (rather than incremental) appearance of metamerism during the evolution of a lineage, and eliminate the need for implausible adaptationist scenarios for its emergence[13].

Differential Adhesion and Segmentation

We can consider whether segmentation fulfills the necessary criteria for being generic to tissues. In most instances, segmental organization appears to be based, in part, on the inability of otherwise similar tissues to exchange cells at their common boundaries or interfaces. This is the case, for example, for the half-somites of the chick embryo[14], the mesodermal compartments of *Drosophila* metameres[15], and the rhombomeres of the vertebrate brain[5]. Immiscibility across tissue boundaries has been analyzed by Steinberg and co-workers in terms of the *differential adhesion hypothe-*

sis[16,17]. In this view, the ability of cells to change position with respect to one another, while remaining bound together in a tissue mass, is seen to contribute to the tissue's 'liquid-like' behavior. And just as distinct liquids will be immiscible if the binding among like molecules is sufficiently stronger than the binding among unlike molecules, tissues can also be immiscible if homotypic adhesive interactions are stronger than heterotypic interactions. There is a great deal of experimental evidence in support of this hypothesis[17]. Moreover, as originally predicted[16], tissues need not use different systems of adhesive molecules to establish immiscibility boundaries. A recent study of forced expression of either L-CAM or N-cadherin in an originally nonadhesive cell type demonstrated that *quantitative* differences in the level of expression of a common adhesive molecule was sufficient to make two otherwise identical cell types sort out into non-mixing domains[18].

Boundaries of immiscibility may also occur in *mesenchymal* tissues in which cells are not directly in contact with one another. Flank and limb bud mesenchyme in chick embryos do not mix with one another[19], and endocardial cushion mesenchyme can be induced to segregate from myocardium by stimulation of extracellular matrix deposition with transforming growth factor β (TGF-β)[20]. It has been suggested that quantitative differences in the density of networks of extracellular matrix fibers may contribute to mesenchymal immiscibility[21].

The presence of at least one type of intercellular adhesion system is a *sine qua non* of multicellularity. Because differences in the level of expression of such a system in different regions of a cell aggregate can be achieved in many ways, immiscibility, and the boundaries that result from it, can be considered to be a generic tissue property.

Spatial and Temporal Periodicities and Segmentation

While immiscibility is a presumed hallmark of any 'developmental compartmentalization'[22,23], segmentation requires, in addition, the linear arrangement of several metameric units. Sequential organization is achieved in at least two distinct ways. In long germ band insects such as *Drosophila*, for example, a series of 'chemical stripes,' consisting of alternating evenly spaced bands of the transcription factors specified by the 'pair-rule' genes *even-skipped (eve)* and *fushi tarazu (ftz)*, arises early during development[24,25], when the embryo is still a syncytium. The first evidence of physical segmentation occurs after cellularization, and is associated with the differential activation by eve and ftz proteins of 'segment polarity' genes such as *engrailed*[26], which is believed to indirectly regulate cell-cell interactions. The expression of *engrailed* occurs in a spatially periodic fashion, reflecting the prepatterns of the activators eve and ftz.

In short germ band insects and crustaceans, no such prepattern is established prior to segmentation. Instead, segmental primordia are added caudally by production of new cells from a subterminal growth zone[2,27]. Interestingly, *engrailed* is produced in a portion of each of these segments in a pattern similar to that in *Drosophila* segments[2]. What might the connection be between these two types of segment-generating mechanism?

A common theme in both of the processes described (in addition to the formation of boundaries across which cells will not mix), is the phenomenon of *periodicity: spatial* periodicity in the long germ band case, and *temporal* periodicity in the short germ band case. In general terms, if a molecule which regulates cell adhesion were to be distributed in a spatially periodic fashion across a tissue, then adhesivity itself would come to vary in a similar fashion, and segmentation would ensue. Similarly, if a regulator of adhesivity were to wax and wane with time in any local region of the tissue in which cells were also multiplying, a series of bands of non-mixing tissue could arise.

The consequences of temporally periodic regulation of adhesivity are less obvious than those of spatially periodic regulation, but an example will illustrate the point (Fig. 1). Consider a synchronized population of cells which divide every three hours. Let us assume that the number of adhesive molecules on the surfaces of these cells is set at the time of mitosis, as a function of the cellular concentration of a regulatory molecule R, and that each cell retains its 'adhesive state' during its lifetime. Let us also assume that the cellular concentration of R oscillates with time, with a period of two hours. If the peak of R coincides with a mitotic event at time 0, then, three hours later, when the next group of cells is generated in the zone of proliferation, R will be in the middle of a cycle; it will return to its peak value only in time for the generation of the third tier of cells, which will thus have identical adhesive properties to the first tier. The production of alternating, non-mixing bands of tissue is a general consequence of this type of mechanism (Fig. 1).

The 'temporal oscillation' hypothesis provides a way of understanding how embryos with very different numbers of cells can generate similar numbers of segments, a phenomenon referred to as 'scale adaptation'[28]. For example, if the concentration of the adhesivity regulator R traverses a fixed number of cycles in a given amount of time, it will mark out a certain number of segments relatively independently of the number of cell cycles traversed during that time. The main effect of reduced embryo size would be to decrease the number of cells per segment.

Let us assume, for instance, that the period of R's concentration oscillation in the growth zone is *longer* than that of the cell cycle, and that a peak of R (the only value that can induce one of the two adhesive states, in this simple example) coincides with mitosis once every fourth cell division. If the cell cycle period was prolonged so that a peak of R now coincided with mitosis every second cell division, the number of segments generated in any given time period would be unaffected, although the number of cells per segment would be decreased.

The case in which the ratio between the period of R and that of the cell cycle is not an integer can have particularly interesting consequences. If the period of R was 9/7 (=1.29) as long as the cell cycle, then a peak of R would coincide with mitosis once every seven cycles of R (assuming the oscillations started in phase). After 35 R-cycles, five repeating units will have formed which, by the assumptions of Fig. 1, would

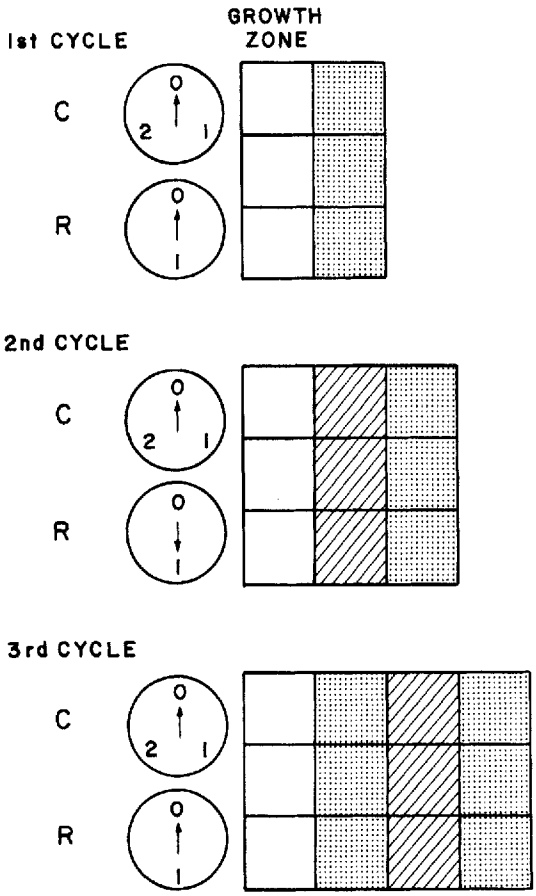

Fig. 1. Model for the generation of segments in a growth zone, by the temporal oscillation of the concentration of a molecule that regulates cell adhesion. The clock faces represent the phase of the cell cycle (C) and that of the periodically varying regulatory molecule (R). It is assumed that the duration of the cell cycle is three hours, the period of the chemical oscillation is two hours, and that both cycles start together. During the first cell cycle, newly formed cells have an adhesive state specified by the initial value of R (stippling). During the second cell cycle, R is in mid-cycle, and the newly formed cells have a different adhesive state (hatching). During the third cell cycle, R is again at its initial concentration, and the new cells have the first adhesive state.

correspond to ten segments. If the cell cycle was now slowed experimentally so that the period of R was 6/5 (=1.2) as long as the cell cycle, then seven repeating units, or 14 segments, would form during the same period. This could possibly account for the result reported by Itow[27], in which horseshoe crab embryos that were exposed to agents that slowed the cell cycle unexpectedly developed extra segments.

Positive Autoregulation and the Biochemical Bases of Temporal and Spatial Periodicities

Is it reasonable to suppose that the temporally and spatially periodic signalling systems discussed here are generic to tissues? Temporal oscillations in metabolites and regulatory molecules, including, but not limited to events of the cell cycle, are well-known[29-31]. Plausible mechanisms for a number of these oscillations have been proposed[32-34] and what they have in common is the presence of positive autoregulation ('autocatalysis'), in the context of otherwise self-limiting kinetics. Biochemical oscillations can thus arise simply from a formal set of regulatory interactions among reacting components; they are not a function of any specific class of molecules. Such oscillations can therefore be considered generic to cells and tissues, in the sense defined earlier.

With regard to spatial periodicities, Turing[35] demonstrated mathematically that if a positively autoregulatory molecule is capable of diffusing away from its source, and its production is inhibited by another diffusible molecule, chemical stripes, spots, and other spatially periodic patterns can arise when certain ratios among the reaction and diffusion coefficients obtain. Chemical systems having these characteristics, that were allowed to react in semi-solid matrices, indeed exhibited such patterns[36,37].

Several molecules that regulate cell adhesivity and extracellular matrix production are positively autoregulatory: eve and ftz proteins[38,39], which regulate *engrailed* in *Drosophila*, and members of the TGF-β family[40], which regulate fibronectin and collagen production in vertebrates, are two cases in point. As a result of positive autoregulation there would be a certain range of kinetic constants for which the concentrations of these regulatory molecules would inevitably be periodic in time. Furthermore, eve and ftz, and TGF-βs, are capable of diffusing away from their points of origin – the former, the *Drosophila* transcription factors, because they are produced in a syncytium, and the growth factors because they are secreted. Assuming diffusible inhibitors are also present, an appropriate balance of production and diffusion rates can lead to spatially periodic distributions of these regulatory molecules.

Adhesion, and temporal and spatial periodicities in the concentrations of molecules which regulate adhesion, are therefore essential or characteristic properties of embryonic tissues that are not tied to any particular class of molecules. For this reason segmentation may reasonably be considered to be a feature that would have emerged in a variety of different settings. If this view has merit, certain developmental and evolutionary consequences should follow.

Modes of Segmental Organization

Two distinct types of segmental organization could arise by the combined action of periodic signalling and differential adhesion, regardless of whether the signalling system is spatially or temporally periodic. If cells produced one amount or type of adhesion molecule during a portion of a cycle, and another during the complementary portion ('square wave' mode; Fig. 2a), a series of metameres would form which would have the predicted property that juxtaposition of tissue from any position within adjacent units would regenerate a physical boundary (i.e., a barrier to mixing), but juxtaposition of tissue from alternate units would not. This result has indeed been observed in grafting experiments with the developing chick hindbrain[5]. Alternatively, if cells produced little or no adhesion molecule at the start of a cycle, and

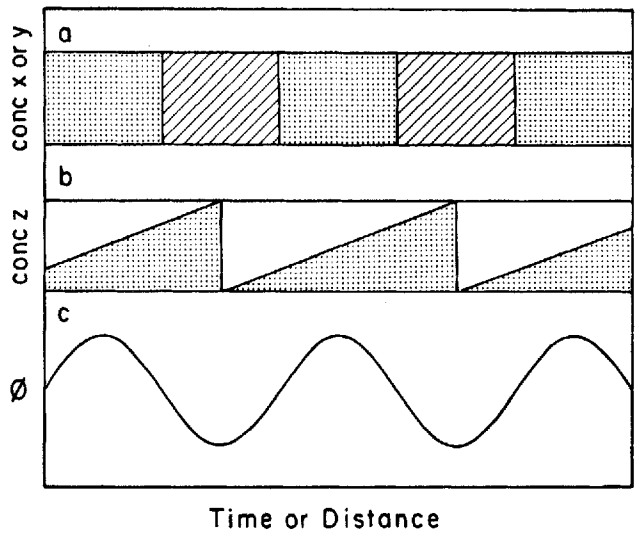

Fig. 2. Schematic representation of two modes of segment generation based on a periodically varying regulatory molecule. In the 'square wave' mode (a), adhesion molecule x (stippling) is synthesized in response to a range of concentrations of the regulatory molecule, and adhesion molecule y (oblique lines) is synthesized in response to the complementary range. (The designations x and y may also represent greater and lesser amounts of the same adhesion molecule). In the 'saw tooth' mode (b), adhesion molecule z (stippling) is synthesized in increasing amounts over the full range of concentrations of the regulatory molecule, and its synthesis is reset to baseline levels at the beginning of a new cycle. The variable ϕ in (c) is the 'phase' of the regulatory cycle, and reflects the concentration of the temporally or spatially varying regulatory molecule.

increasing amounts as the cycle progressed ('saw tooth' mode; Fig. 2b), the resulting metameres would be predicted to have the property that nearby tissues from opposite sides of a boundary would regenerate the boundary when juxtaposed, but tissues from *corresponding* positions in adjacent segments would fail to regenerate a physical boundary. This result was seen in boundary extirpation experiments in the insect *Oncopeltus*[41]. Whereas the removal of the segment boundary resulted in regeneration of a new boundary, removal of an entire segment length from halfway down one segment to halfway down the next did not lead to new boundary formation.

The model represented in Fig. 2 attributes segmentation solely to differential adhesion and periodic regulation of adhesivity. It provides a framework, however, for accounting for segmental properties as disparate as those seen in long and short germ band insects, and for differences between the results of boundary regeneration experiments in the chick hind brain and the insect cuticle.

This framework can help explain even more complicated experimental outcomes. In lineage marking experiments with chick somites it was found, unexpectedly, that the progeny of a single cell was able to cross the intrasegmental boundary within a given somite, or the intersegmental boundary between two somites, but not both boundaries[3]. This result can be accounted for by the model shown in Fig. 2 by assuming that somites consist of at least two different cell populations[14], utilizing distinct adhesion systems. If each set of adhesion molecules is regulated according to the square wave mode, with a quarter period offset in the respective (spatially or temporally) periodic regulatory signals, then boundaries of immiscibility would be created for one cell population at the segmental border, and for the other population *within* each segment. Any given marked cell, however, would only abide by one of these boundaries.

Stabilization of Generically-Templated Segmental Patterns

Pattern-forming mechanisms based on chemical kinetics, with or without diffusion, are inherently temperature-sensitive. In the case of reaction-diffusion mechanisms they would also be sensitive to the spatial scale of the system. If the view presented here is valid, therefore, it would be expected that the number of metameres in the earliest segmented forms would have been developmentally variable. Any forms that were physiologically and ecologically viable would have been under selective pressure to evolve additional mechanisms to ensure that development 'bred true'. Evolution that reinforces a particular outcome, rather than leading to phenotypic change, has been called 'stabilizing'[42] or 'canalizing'[43] evolution.

Assuming, for example, that a striped concentration pattern of a positively autoregulatory transcription factor had arisen by a reaction-diffusion process, the pattern could have been stabilized and reinforced by mechanisms that tied the production of the stripes to other molecular cues present in the system. The regulation of *eve* and *ftz* in *Drosophila* may represent such a situation, for these factors, in addition to being positively autoregulatory, are also regulated by nonuniformly distributed maternally deposited factors, as well as products of the 'gap' genes, expressed from the zygotic genome, in a concentration-dependent fashion[1,44,45]. I suggest that these regulatory circuits may have been evolutionarily selected for their capacity to increase the reliability of production of viable striped patterns originally templated by the coupling of diffusion with positive autoregulation. The alternative notion seems less likely: that the identical-appearing, uniformly spaced, eve or ftz stripes arose one by one during evolution, by the gradual acquisition of the dedicated promoters, sensitive to unique complexes of *trans*-acting factors, seen in modern *Drosophila*.

Similarly, the overall organization of the vertebrate limb, with its two-fold segmentation of skeletal tissues, may have been templated during tetrapod evolution by a generic mechanism, such as a reaction diffusion process involving TGF-β[46-48]. But the reliable control of the size and number (and identity, see below) of skeletal elements in the limbs of modern vertebrates may be the result of a subsequently evolved link between the action of the 'core' patterning mechanism[49] with homeobox gene products[50,51] that were nonuniformly distributed in the limb-forming tissues for incidental reasons.

Reaction-diffusion mechanisms are notoriously poor at producing the same pattern over a range of spatial scales:

segment number, for example, would tend to increase with increase in tissue length. Any organism that originally depended on such a process for the generation of metameres would have been under intense selective pressure to evolve to a state in which segment number came to depend on landmarks other than ratios of reaction and diffusion rates. Simple gradients, which can span the same concentration range over different distances, are ideal landmarks for building scale adaptation into a developing system. They are poor candidates, however, for the primordial initiators of segmentation, because a sequence of finely-tuned thresholds of gene response is required in order to generate a regularly-spaced, alternating series of on and off states using a gradient. Hybrid gradient/reaction-diffusion mechanisms have been considered previously in connection with the sequence of segmentation-related gene activation events in the early embryos of modern *Drosophila*[52]. But the order in which molecular components were recruited during phylogeny need not reflect the order in which they are used during ontogeny. The possibility suggested above, that gradients were co-opted to reinforce and stabilize particular segmental patterns that were originally templated by a generic process, provides a scenario for how the biologically useful properties of these systems could have become integrated during evolution.

These ideas may be tested experimentally by producing null mutations in genes hypothesized to play a 'reinforcing' rather than 'core' role in segmentation. The prediction would be that for many such cases, the segmental pattern would be relatively unaffected, but its sensitivity to external factors, such as temperature, might be enhanced. Caution must be observed in interpreting such knock-out experiments, however, since a 'reinforcing circuit' might actually depend on a balance between two gene products. The interaction between *nanos* and *hunchback* in the *Drosophila* embryo may provide a relevant example. The gene product of *nanos* apparently serves only to repress the translation of mRNA specified by the maternal *hunchback* gene[53]. Embryos that lack both maternal *hunchback* gene product and a functional *nanos* gene develop normally, but if *nanos* alone is knocked out posterior segments fail to form and the embryos die[54].

Segment Identity

If 'incidental', nonuniformly distributed transcription factors can plausibly have been recruited for the stabilization and reinforcement of segment number, such factors can equally well be used to create individual identities for originally equivalent segments. Thus, homeobox-containing genes of the Antennapedia and Bithorax complexes, believed to be evolved from a single homeotic complex (HOM-C), are expressed in partially overlapping domains along the anteroposterior axis of the *Drosophila* blastoderm[55], and their homologues are analogously expressed along the rostrocaudal axis of the vertebrate embryo[56], and within the developing vertebrate limbs[50,51]. The regulatory basis for the spatial arrangement of these expression domains may depend on their unusual, evolutionarily conserved genomic organization[57], but it is apparently not specifically tied to segmentation, since a similar genomic arrangement of HOM-C-related genes occurs in the nematode, *C. elegans*[58], which is not segmented and is not thought to have a segmented ancestor[8].

What does unify all known cases, including *C. elegans*[59], is that differential expression of HOM-C-related genes, which encode transcription factors, endow cells with distinct properties. These variable properties can be based on ordinary cell functions, such as regulation of amounts of cell adhesion molecules[60]. When expression boundaries of such genes coincide with segmental boundaries, as is the case in some *Drosophila* segments[55], and in the rhombomeres of the vertebrate hindbrain[61], segments will seem to be 'defined' by this expression, even if the 'segment identity' genes had nothing to do with the mechanism by which metameres were generated. The independence of initial segment formation from segment diversification seems to be the case during *Drosophila* embryogenesis[1,62,63], where the expression of the HOM-C genes is superimposed upon an earlier established prepattern of pair-rule (i.e., *eve* and *ftz*) stripes and the alternating bands of engrailed protein induced by the pair-rule pattern. Analogously, the identity, or at least the physical appearance, of digits in the developing chick limb can be modulated by misexpression of *Hox-4.6 (HoxD-11)*, a member of a family of avian HOM-C homologues[64]. It is significant that despite the transformations, the limbs still contain discrete, evenly spaced digits. This suggests that digit number is controlled by a process different from that regulating digit identity[7,46].

I suggest that the linking of segmentation mechanisms to nonuniformly distributed modulators of cell phenotype occurred several times throughout evolution. The modulatory gradients may have been phylogenetically conserved for reasons having nothing to do with segmentation. In particular, the HOM-C genes in insects, and their homologues in other taxa, may have been under common evolutionary pressure to maintain their general spatiotemporal order of expression because of their integration into a variety of systems requiring spatial diversity. If, as proposed above, segmentation is generic to tissues, with many possible underlying molecular mechanisms, the involvement of HOM-C-related gene products in the generation of metameres in widely disparate taxa (such as BX-C in *Drosophila* body segments[53] and the Hox-2 family in mouse hindbrain rhomobomeres[59]) need not represent evidence for the origin of this patterning process in a common ancestor. (Indeed, the common ancestor of vertebrates and arthropods is believed to have been unsegmented[9,10].) An alternative possibility must also be considered: that such molecules may have proved suitable for repeated recruitment into mechanistically, rather than genealogically, homologous segmentation processes.

Conclusions

Because an appropriate causal link between *any* periodically varying signal molecule and the synthesis of *any* molecule affecting intercellular adhesion is sufficient to produce segmentation, it is not surprising that the metameric theme and its variations are so prevalent over the course of evolution. It is generally accepted that segmentation arose independently

in at least five lineages[8-10]; if segmentation is indeed a generic property of tissues it becomes unnecessary to postulate gradualist scenarios for its evolution. Disparate segmented body plans and organs could have typified many early lineages, with natural selection culling them out, rather than generating them by increments.

Both temporal and spatial periodicities in the concentrations of tissue molecules can readily emerge when one or more of the molecules are positively autoregulatory. The possibility of a common generic component to what would otherwise appear to be two distinct mechanisms for segmentation[65], helps account for why apparently dissimilar segmentation processes occur in different kinds of insect, and even within different regions of the vertebrate axis[28].

Previous work has suggested that, depending on the case, adjacent segments can be 'nonequivalent', differing from each other in uniformly expressed segment-specific properties[5], or they can be 'equivalent', but internally differentiated by a spatially graded property[41]. The discussion presented here suggests that the explanation for this difference in segmentation modes need not lie in the periodicity-generating mechanism, or in the adhesive mechanism, *per se,* but in the relationship between the two (see Fig. 2).

The individual gene products that would typically be involved in segmentation processes, either as part of generic adhesive or periodic signalling functions, or as nonuniformly distributed modulators of cell phenotype, would not be expected to be intrinsically segmentation-related, but would have other roles in other contexts. The view presented here suggests criteria for decomposing complex genetic networks, such as those involved in establishing the segmental pattern of the insect body, or the skeleton of the vertebrate limb, into subsets of circuits with specific roles: establishment of chemical periodicities, regulation of adhesivity, reinforcement and stabilization of pattern, and diversification of cell and segment function. These generic functions may be served by different or similar sets of molecules in various lineages, and taken together, may constitute a portion of the 'grammar' of metazoan organization.

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