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Segmentation in frogs

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Summary

This paper reviews evidence relating to the question, at what stage in the development of the frog embryo are segment boundaries specified? Current evidence leads to the hypothesis that a spatiotemporal series of cell states leading to segmentation is continuously initiated at a position 200 to 300 μm from the posterior end of the presomitic mesoderm, about nine somite intervals before the formation of a definitive somite. The evidence suggests, though by no means proves, that segment boundaries are specified close to this time. This hypothesis relies critically on evidence concerning the effects of disruptive agents, the extent of cell mixing prior to the early gastrula stage, fate-map data, and a comparison with development in the mouse where a similar fate map can be related to morphological evidence of somitomeric segmentation.

Evidence regarding the organization of the posterior, undifferentiated zone of the mesoderm in the frog embryo indicates that the cells are not proliferating rapidly, but are undergoing cell movements and rearrangements associated with caudal extension. The speculation that the segment pattern derives from inductive interactions in this region is discussed.

Key words: segments, development, vertebrate, Amphibia, mesoderm.

Introduction

Pattern formation is essentially a matter of integrating the genetically determined activities of individual cells. This view implies that there exists, at the tissue level, some kind of framework for cell co-ordination (Wolpert, 1971; Meinhardt, 1986). The current, genetically-based work on *Drosophila* is beginning to uncover elements of this framework in insects, as reference to many of the papers in this volume will show. Evolutionary history suggests, however, that segmentation arose separately in the vertebrates and invertebrates. In contrast to the development of insects, almost nothing is known about the spatial and temporal coordination of the process of segmentation in vertebrate animals.

Several general reviews of segmentation in vertebrates have recently been published, for example in the compendium edited by Bellairs, Ede & Lash (1986) (see also, Cooke (1981) for an overview). The present paper reviews evidence relating to a single question. At what stage in development do the cells that will populate adjacent segments become isolated, or differentiated, from one another: that is, when are segment boundaries specified? I have focused attention on this particular question for two reasons. First, the answer is clearly of crucial importance to anyone approaching an experimental analysis of the mechanism of segmentation, and is thus particularly topical in view of the general expectation that current molecular techniques may soon open novel approaches in this field. Second, although aspects of this question have been addressed experimentally, the current evidence has not, to my knowledge, been drawn together in a review. There appears now to be sufficient evidence to suggest, though with more precision than certainty, the place in the embryo where segment boundaries are specified and to point to selected aspects of tissue organization in this region which favour some hypotheses regarding the mechanism of segmentation and place constraints on others. Much of the evidence comes from experiments on the frog, but I will take the view that the essential features of vertebrate segmentation have been conserved so that evidence can be drawn from work on the fish, chick and mouse. Indeed, an important point that will emerge is the similarity between the organization of the segmenting tissues in different vertebrates.

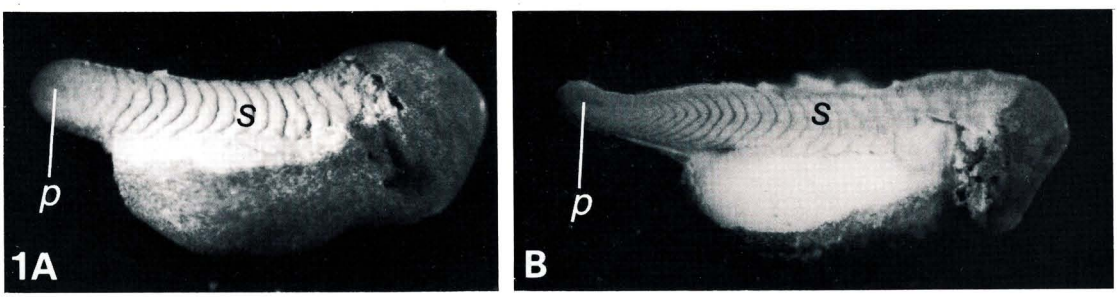

Fig. 1. Segmentation in the frog embryo. (A,B) *Rana* embryos fixed and partially stripped of their skin to show the developing somites and presomitic mesoderm. (A) 13-somite stage. (B) 22-somite stage. Abbreviations; *s*, somites; *p*, presomitic mesoderm. ×13·5; bar, 1 mm.

When are segment boundaries established?

From an experimentalist's point of view, the predominant feature of vertebrate segmentation is that it proceeds sequentially from head to tail (Fig. 1). Such evidence as we have regarding the organization of the tissue that is yet to form segments, as well as that in which the early stages of segmentation are already visible, indicates a spatiotemporal gradient of differentiation. Successive stages of segment development are simultaneously represented in each embryo, laid out from the tail towards the head.

This situation has influenced current thinking in two ways. First, it has engendered a dynamic, process-based, view. Second, it has led to the hypothesis that a common developmental programme leading to segment formation is periodically initiated at some position posterior to the visible wave of segment formation. This hypothesis underpins almost all current work on the mechanisms of segmentation. *Drosophila* genetics – in particular, the patterns of expression and mutant phenotype of the pair-rule and segment-polarity genes (see Akam, 1987 for a review) – has provided powerful evidence that the same set of genetic interactions is repeated along the embryo in the formation of successive segments, though here the unit of repetition is a pair of segments and the anteroposterior dynamics appear to be telescoped so that all the segments form almost simultaneously. Evidence in support of the hypothesis in vertebrates is much more superficial. In the frog, assays of homology in the development of successive segments rely on rather nonspecific criteria of morphology, kinetics and response to disruptive agents. As genes involved in vertebrate segmentation become identified, it will be important to map their expression, by *in situ* hybridization or immunohistochemistry, in order to explore this situation more fully. For the present purpose let us accept the idea that a serially repeated programme allocates tissue to successive segments and merely note two additional uncertainties. First, it is not clear whether the spatiotemporal differentiation of the prospective segmental tissue precedes, or derives from, this periodic process. Second, it is not certain that the segments we see are homologous to the original units of segmentation in the hidden earlier stages of development (for a discussion of a similar problem in insects, see Lawrence in this volume). With this background of uncertainty, let us focus our attention on the frog embryo during the time when it is forming its segments and, in particular, on the development of the presomitic mesoderm, the sketchily charted territory that stretches backwards from the last-formed visible segment (Fig. 2A). How far back does the spatiotemporal gradient of differentiation extend? Where, along this gradient are segmental boundaries established?

The frog has particular advantages for the investigation of these questions. The *Rana* embryo forms about 40 somites over 4 days (at 15 °C). The interval between the formation of successive somites is a constant at any particular temperature (in *R. temporaria*, 2 h 20 min. at 15 °C (Elsdale & Davidson, 1987)). For the investigator, this 'somite interval' performs the function of a clock, allowing steps in the process of segment development to be located in time as well as in space. Experimental exploitation of this situation is favoured by the accessibility of frog embryos, by comparison with those of birds and mammals, and the additional advantages of natural spawnings of *Rana temporaria* which, by comparison with induced ovulations of *Xenopus*, produce more uniform batches of larger, more slowly developing embryos.

The visible landmark in the process of segmentation in the frog is the formation of the segmental block of mesodermal tissue, the somite (Hamilton, 1969; Youn *et al.* 1980). The last-formed definitive somite is generally regarded as the most posterior one that is clearly demarcated on the outer mesodermal surface of the fixed embryo stripped of its skin (Fig. 1). Posterior to the last-formed somite, morphology provides only limited indications of segmental organization. Following enzyme-aided dissection,

two or three additional intersegmental furrows are evident on the inner surface of the mesoderm, indicating a morphogenetic region encompassing about three somites-worth of tissue (Davidson, unpublished results). Beyond this region, the presomitic mesoderm of the frog presents no hint of segmentation (Youn & Malacinski, 1981). In many vertebrates, however, periodic cell patterns – somitomeres – that suggest segmental organization are discernible in the presomitic mesoderm under the SEM (Meier, 1979). These are difficult to detect. However, a comparison of the morphology of one side of the embryo with the development, in culture, of the other side suggests that each somitomere forms one somite (Packard &

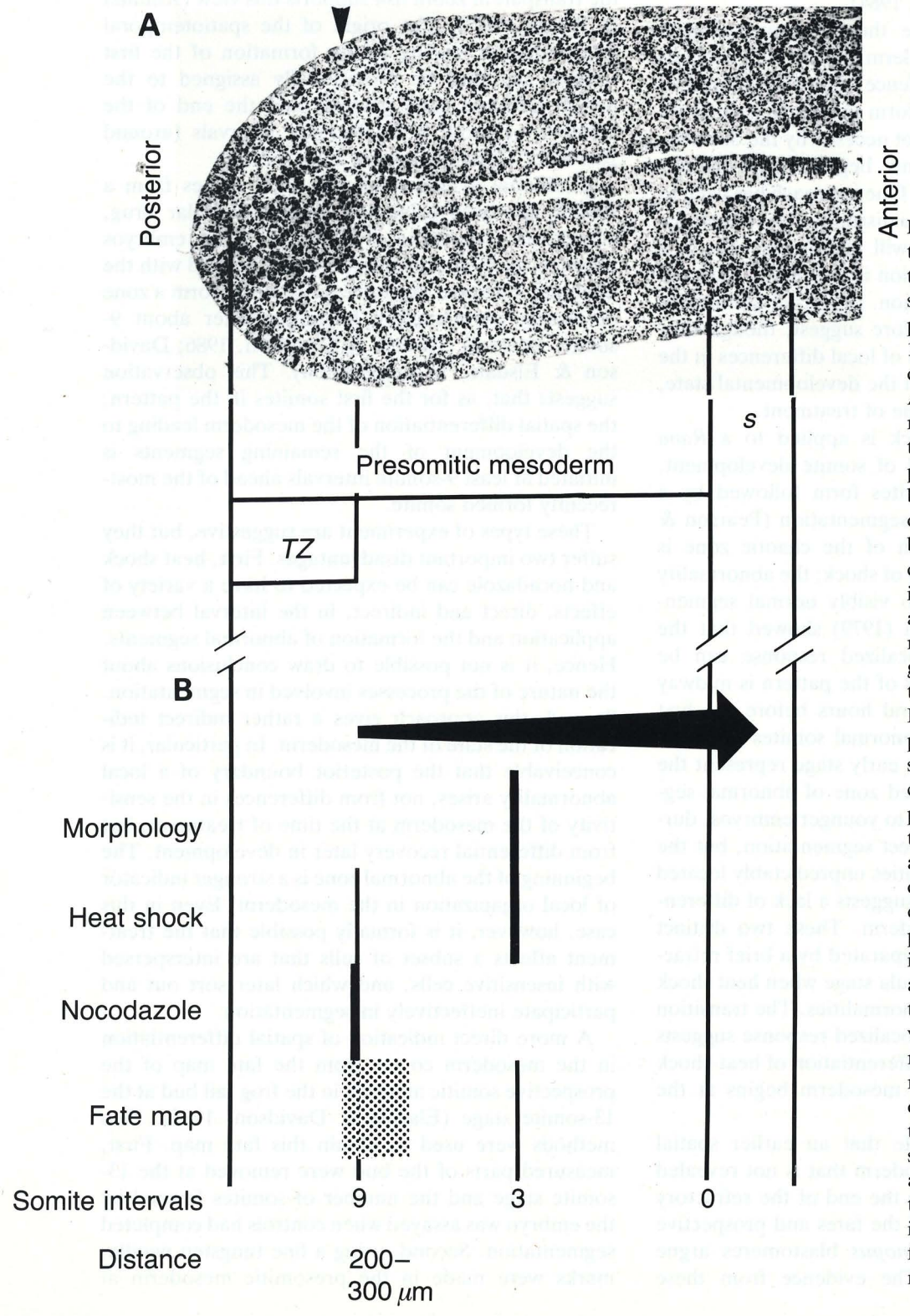

Fig. 2. The organization of the presomitic mesoderm in the frog embryo. (A) Frontal section through the tail and posterior trunk region at the 13-somite stage, at the level of the notochord. Abbreviations: *s*, the most recently formed somite; *TZ*, the terminal zone of the presomitic mesoderm. ×100; bar, 100 μm. (B) A summary of the approximate relation between the different regions of the presomitic mesoderm identified by different approaches. The large arrow represents a spatiotemporal gradient of differentiation in the presomitic mesoderm. Each part of the tissue progresses through a series of states. The series is continuously initiated posteriorly and culminates in the formation of somites anteriorly. In the lower part of the diagram the spatial differentiation in the presomitic mesoderm, as indicated by the different approaches discussed in the text, is represented by vertical bars. The temporal relations between these regions are indicated in terms of somite intervals prior to the formation of a definitive somite. The approximate anterior boundary of the terminal zone (small arrow), is also located by distance from the tip of the tail-bud mesoderm.

Meier, 1983; Tam, 1986). Six or seven somitomeres have been observed in the mouse (Tam *et al.* 1982) and snapping turtle (Packard & Meier, 1984), thirteen in birds (Packard & Meier, 1983). Somitomere-like structures have also been described in the cephalic region of the newt where stable somites do not form, suggesting a primitive segmentation in this region (Jacobson & Meier, 1984).

Experiments that probe the non-morphological organization of the mesoderm in the frog embryo provide independent evidence that the differentiation of tissue destined to form segments at different levels of the axis, though not necessarily the differentiation of individual segments, begins almost one day ahead of somite formation. One approach has been to apply to the whole embryo a disruptive treatment, for example heat shock, that will affect only cells in a sensitive state. The disruption is eventually reflected in abnormal somite formation. Localized abnormalities of segmentation therefore suggest, though they do not prove, the existence of local differences in the sensitivity, and therefore in the developmental state, of the mesoderm at the time of treatment.

After a brief heat shock is applied to a *Rana* embryo during the period of somite development, two or three normal somites form followed by a discrete region of chaotic segmentation (Pearson & Elsdale, 1979). The length of the chaotic zone is proportional to the severity of shock; the abnormality is followed by a return to visibly normal segmentation. Elsdale & Pearson (1979) showed that the earliest time that this localized response can be induced in the first somites of the pattern is midway through gastrulation, several hours before the first somites become visible: abnormal somites observed in embryos shocked at this early stage represent the posterior end of a truncated zone of abnormal segmentation. Shocks applied to younger embryos, during early gastrula, also affect segmentation, but the result – sporadic abnormalities unpredictably located over the first 25 somites – suggests a lack of differentiation in the early mesoderm. These two distinct periods of sensitivity are separated by a brief refractory period at the midgastrula stage when heat shock does not induce somite abnormalities. The transition from a disorganized to a localized response suggests that the anteroposterior differentiation of heat-shock sensitive processes in the mesoderm begins at the midgastrula stage.

Of course, it is possible that an earlier spatial differentiation of the mesoderm that is not revealed by heat shock exist before the end of the refractory period. However, maps of the fates and prospective axial specifications of *Xenopus* blastomeres argue against this possibility. The evidence from these studies is by no means clear-cut, but it appears that enough cell mixing occurs before the early gastrula stage to make it unlikely that any fine-grained anteroposterior differentiation of the mesoderm would persist through gastrulation. (Cooke & Webber, 1985*a*,*b*; Cooke, 1985; Dale & Slack, 1985*a*,*b*; Moody, 1987). Direct observation of marked cells in the transparent zebra fish supports this view (Kimmel & Warga, 1987). The origin of the spatiotemporal differentiation leading to the formation of the first somites can thus be provisionally assigned to the period between early gastrula and the end of the refractory period, 8- to 9-somite intervals (around 20 h at 15 °C) before somite formation.

Additional evidence for this view comes from a similar approach using the antimicrotubular drug, nocadazole (Hoebeke *et al.* 1976). Frog embryos between the 7- and 14-somite stages, treated with the minimum effective exposure to the drug, form a zone of chaotic segmentation beginning after about 9-somite intervals (Elsdale & Davidson, 1986; Davidson & Elsdale, in preparation). This observation suggests that, as for the first somites in the pattern, the spatial differentiation of the mesoderm leading to the development of the remaining segments is initiated at least 9-somite intervals ahead of the most-recently formed somite.

These types of experiment are suggestive, but they suffer two important disadvantages. First, heat shock and nocadazole can be expected to have a variety of effects, direct and indirect, in the interval between application and the formation of abnormal segments. Hence, it is not possible to draw conclusions about the nature of the processes involved in segmentation. Second, this approach gives a rather indirect indication of the state of the mesoderm. In particular, it is conceivable that the posterior boundary of a local abnormality arises, not from differences in the sensitivity of the mesoderm at the time of treatment, but from differential recovery later in development. The beginning of the abnormal zone is a stronger indicator of local organization in the mesoderm. Even in this case, however, it is formally possible that the treatment affects a subset of cells that are interspersed with insensitive cells, and which later sort out and participate ineffectively in segmentation.

A more direct indication of spatial differentiation in the mesoderm comes from the fate map of the prospective somitic material in the frog tail bud at the 13-somite stage (Elsdale & Davidson, 1983). Two methods were used to obtain this fate map. First, measured parts of the bud were removed at the 13-somite stage and the number of somites formed by the embryo was assayed when controls had completed segmentation. Second, using a fine tungsten needle, marks were made in the presomitic mesoderm at

measured distances from the tip of the tail bud and later identified in the completed somite pattern. Such a fate map allows the observer to predict approximately which parts of the mesoderm will contribute to which somites: it indicates the way the prospective pattern is packed without, in itself, implying the existence of any segmental organization. When this map is related to the changing shape of the mesoderm we can gain additional insight into the spatial differentiation of the tissue. The presomitic mesoderm appears, on morphological evidence, to be extending uniformly throughout its length: extension is accompanied by a uniform narrowing in the dorsoventral and mediolateral dimensions (Elsdale & Davidson, 1983; see also, Fig. 1). Any pattern of differentiation already present in this tissue would be expected also to extend: the fate map would show either a uniform spacing between prospective segments or at least one which increased uniformly towards its anterior end.

In fact, prospective segments are not uniformly packed in the fate map (Elsdale & Davidson, 1983). There is an abrupt change in the measured packing near the posterior end of the mesoderm. Anterior to this position, the prospective pattern undergoes an approximately eightfold extension until each prospective segment attains the width of a newly formed somite close to the region where somitogenesis becomes visible on the inner mesodermal surface. In the 13-somite embryo, this 'zone of extension' contains the material for about six segments, the 'morphogenetic zone' material for about three. The remaining twenty, or so, somites derive from a proportionately much smaller region, approximately the posterior one-third of the presomitic mesoderm, which we may call the 'terminal zone'. The absence of any evidence for extension of the prospective pattern in this region, despite the obvious extension of the tissue itself, suggests that this part of the mesoderm is undifferentiated with respect to its anteroposterior position. In addition, the large number of segments derived from this material makes it difficult to see how any segmental boundaries could be specified in this region.

Evidence from this approach therefore suggests that cells, after passing out of the terminal zone of the presomitic mesoderm, are imprinted with some local quality that makes future segment boundaries approximately predictable to the observer. The map locates the approximate boundary of the terminal zone as a measured distance (200–300 μm) from the posterior end of the mesoderm and as a number of prospective segments (about $6+3=9$) posterior to the last-formed definitive somite. These estimates can only be rough guides because the resolution of the method is low in the region of the map where the density of prospective segments is high. However, it is clear that the boundary of the terminal zone coincides rather closely with the transitions in tissue state that were tentatively identified using heat shock and nocadazole (Fig. 2B).

The state of differentiation of the mesoderm in the zone of extension is unclear: the predictability of segment boundaries outside the terminal zone may reflect one of two situations. One possibility is, of course, that segments become differentiated as cells leave the terminal zone. The second is that some quality is imprinted on the tissue that will, closer to the time of somite formation, determine the approximate location of segment boundaries. An eloquent example of the latter possibility is the Clock and Wavefront model proposed by Cooke & Zeeman (1976). According to this model, the time at which cells can partake in segment formation is set in a continuous anteroposterior gradient; hence, the development of cells at any particular location can be visualized as a wavefront of change moving towards segmentation. A periodic pattern is formed from this continuous organization because the passage of the wavefront is gated by a second temporal component which functions as a clock: cells can only participate in segmentation in a particular phase of a physiological cycle. (It is assumed that the segment formed during the preceding cycle is refractory to the incorporation of new cells.) Alternatively, segmentation could be brought about by discontinuous mechanical instabilities which arise as a result of the continuously changing morphogenetic properties of the cells and the extracellular matrix (Oster *et al.* 1983; Bellairs, 1979; Hatta *et al.* 1987; Duband *et al.* 1987).

The only evidence that allows us to choose between these two broad possibilities comes from studies on the mouse, which relate the fate map of the segment pattern to morphological evidence of segmentation provided by the detection of somitomeres. Using the deletion approach, Tam (1986) showed that, at successive stages of development, the fate map of the presomitic mesoderm in the mouse embryo is similar to that of the 13-somite frog embryo. Posterior to a region where the prospective material for the next few (5–7) segments is extending, the posterior 200–300 μm of the mesoderm contained the material for the remainder of the pattern. The significant point here is that each of the prospective segments anterior to the terminal zone could be located by its measured position on the fate map and identified with a somitomere detectable under the scanning electron microscope. This evidence supports the view that segment boundaries are formed by the time cells leave the terminal zone. The recent demonstration that labelled cells can move between existing somitomeres (Tam & Beddington, 1987) raises the possibility, however, that the incipient segmental organiz-

ation may retain some fluidity during the somitomeric phase.

In summary, the current evidence leads us to the hypothesis that the spatial differentiation of the mesoderm leading to the formation of segments at successive axial levels begins between 200 and 300 μm from the posterior end of the presomitic mesoderm, about six-somite intervals before the onset of visible somite morphogenesis. Segmental boundaries are probably established at, or close to, this time. This hypothesis relies critically on limited evidence of cell mixing prior to the early gastrula stage, on low-resolution fate-map data, and on the identification of somitomeres in the mouse embryo. It will be important to obtain more detailed evidence, for example of the extent of cell mixing in vertebrates other than fish, specifically aimed at testing this hypothesis. A direct test may be possible in the future if probes become available to map the expression of genes specifically involved in the early stages of segmentation in vertebrates. According to the argument outlined above, such a map would distinguish up to nine presomitic segments posterior to the last-formed definitive somite in *R. temporaria*. In contrast, *Bufo vulgaris*, which also shows a change in sensitivity to nocadazole around 20 h ahead of the formation of the last-formed definitive somite, forms somites at longer intervals (about 4·5 h at 15 °C). In this case, expression in four or five presomitic segments would be expected.

Tissue organization in the terminal zone

The existence of a terminal zone may be general in the vertebrates for it has been defined, by fate mapping, in a small number of widely different species. In addition to the studies on the frog and mouse mentioned above, a similar zone 200–300 μm long has been identified in the axolotl (Armstrong & Graveson, 1988). Tam (1986) has suggested that the chick primitive streak may be equivalent to the caudal tissue (terminal zone) in the mouse. Packard & Meier (1984) have suggested that, in all amniotes, the segmental pattern is defined in the region of Henson's node and the cranial part of the primitive streak. Bellairs has postulated, however, that two distinct components of the posterior tissue contribute to somite formation in the chick. According to this view, small groups of cells that may form discrete foci for segmentation, lie in a region around Henson's node and are drawn out by the node as it moves caudally into the streak. At the regressing node, larger numbers of presomitic cells leave the primitive streak to join these groups in the formation of somitomeres (see Bellairs, 1986 for a review). Hence, the segmental 'pre-pattern' is compressed, but nonetheless differentiated into the precursors of segmental units in tissue immediately posterior to the somitomeric region, while more posterior tissue (the primitive streak) is unpatterned. This view thus attributes a two-phase structure to the 'terminal zone' and carries implications for the mechanism of segmentation different from those explored below. There is, as yet, no evidence to suggest how this prepattern might be established.

In Amphibia, the tissue at the posterior end of the mesoderm – roughly the terminal zone of the fate map – shows no morphological evidence of histodifferentiation, at least at the light microscope level (Elsdale & Davidson, 1983). The notochord becomes differentiated approximately at the anterior margin of the terminal zone. According to Armstrong & Graveson (1988), the mesoderm in the terminal zone of the axolotl comprises loosely organized mesenchyme while the anterior presomitic mesoderm forms a cohesive sheet. The undifferentiated nature of the terminal tissue is further indicated by the observation that its fate is not restricted to somitic mesoderm. For example, cells from the caudal mesoderm have been found to participate in the formation of a variety of tissues in the mouse (Tam, 1984).

The fact that such a short, apparently undifferentiated, region at the end of the mesoderm can generate a large proportion of the segment pattern might suggest that the cells are rapidly dividing. However, in the frog there is only a low level of cell proliferation in the presomitic mesoderm – including the terminal zone – throughout much, perhaps all, of the period of segmentation. We have found that, in histological sections of both untreated, and nocadazole-treated, embryos the mitotic index is too low to suggest a true proliferative zone (an average mitotic index of 20 % throughout the presomitic mesoderm and a maximum of around 30 % in the middle of the zone of extension, after six-somite intervals in 1 μg ml^{-1} nocadazole; Davidson & Elsdale, 1986 and unpublished results). This view is supported by direct counts of cells in the entire presomitic mesoderm at successive stages of development up to the 22-somite stage. These show a cell-doubling time of around 10-somite intervals between the 4- and 14-somite stages; this figure includes a probable contribution from cell recruitment into the mesoderm over the first half of this period (Davidson & Elsdale, in preparation). In addition, we have examined the effect of a temporary (>90 %) inhibition of cell division by X-irradiation at the 13-somite stage. This treatment did not delay or prevent extension of the mesoderm or the formation of a complete complement of somites (>35), though these had only about half the number of cells compared with somites in untreated sibling controls

(Davidson & Elsdale, in preparation). Though proliferation may play a role in the process of segmentation in special cases, these observations appear to rule out general models of segmentation based on proliferation of, for example, stem cells.

The terminal zone, like the rest of the presomitic mesoderm, undergoes active morphogenetic movements associated with the extension of the embryo (Elsdale & Davidson, 1983). The presomitic mesoderm extends during the development of the trunk and early tail somites in *Xenopus* as a result of dorsal convergence movements (Keller, 1976). In addition, histological evidence suggests that, up to about the 10-somite stage, cells are recruited into the mesoderm from the deep neurectoderm (Cooke, 1979). The cell movements that drive the extension of the presomitic mesoderm at later stages have not been examined.

The picture that emerges from these observations is of a small region in which the prospective somitic tissues are as yet undifferentiated and are undergoing cell movements and rearrangements. The framework of coordination that is required to generate a periodic pattern from this tissue must include some means of imposing polarity and discontinuity as well as ensuring differentiation as 'dorsal'-type mesoderm. As a consequence of its small size, the terminal zone is close to several other tissues which could, for example, play inductive roles. Additionally, the tissue is about the right size to accommodate diffusion gradients.

The possibility that segmentation requires inductive tissue interactions has long been recognized. However, much of the experimental evidence for, or against, such a view has focused attention on interactions anterior to the terminal zone. Half a century ago there was controversy over the issue of whether the undifferentiated caudal region of the tail bud represented a blastema of multipotent cells, an independent site of 'secondary morphogenesis' (see Pasteels (1939) for a review). It is now clear that morphogenesis of the tail is essentially a continuation of trunk development and that much of the somitic mesoderm is at least partly specified by inductive interactions occurring in the pregastrula stage. The possibility remains, however, that these early events are only the beginning of a series which may be carried through locally in the terminal zone, the later inductions being crucial for the establishment of the metameric pattern. We might speculate, for example, that mesodermal induction similar to that which occurs much earlier in development (Dale & Slack, 1987*a*,*b*; Cooke *et al.* 1987; Weeks & Melton, 1987; Kimelman & Kirschner, 1987) also occurs in the terminal zone of the mesoderm at the neurula stage to promote or maintain dorsal mesodermal differentiation, leading to the development of notochord and somites. Both endoderm and ectoderm are close to the prospective mesodermal tissue in this region embryo (Elsdale & Davidson, 1983) and may play a role in inducing mesodermal differentiation. In addition, these tissues may, conceivably, impose polarity on the more mobile mesoderm. Inductive interactions within the mesoderm may play a role in structuring or patterning the tissue (see, for example, Cooke *et al.* 1987). It is conceivable that this type of interaction plays a part formally similar to the interaction between domains of cardinal gene activity in the early *Drosophila* embryo, to generate segment boundaries at the anterior margin of the terminal zone. In this context it is of interest that the homeobox containing gene, *Xhox-36*, is expressed in the posterior tissues of the neurula-stage frog embryo (Condie & Harland, 1987).

The work on *Rana* on which this review is based was initiated, and in large part, carried out by Tom Elsdale. It is a pleasure to thank him for many years of help and encouragement. I would also like to thank Jonathan Cooke and the other organizers of this conference for inviting me to speak and Jonathan Bard for help with the manuscript.

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