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Heat shock causes repeated segmental anomalies in the chick embryo

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Summary

A single heat shock, given to 2-day-old chick embryos, can generate multiple but discrete somite and skeletal anomalies. Each of these anomalies is restricted to one, or at the most two, consecutive segments. The anomalies are separated from each other by a distance of 6–7 somites or vertebrae, or a multiple of this distance. These results argue against the 'clock and wavefront' model; while they support the idea of a cellular clock, they are not consistent with a single propagating wave gating cells destined to form each segment.

Heat shock also alters the size and number of segments, as well as the rostrocaudal proportions of the sclerotome. The results are consistent with the rostrocaudal fate of sclerotome cells being determined during segmentation. From our observations, we speculate on the implications for regionalization of the vertebral column.

Key words: chick embryo, heat shock, segmentation, anomaly, clock and wavefront model, vertebral column, biochemical clock.

Introduction

In vertebrate embryos, segmentation of the body plan is most obvious in the pattern of somites, which are laid down in an orderly rostrocaudal sequence. The somites form as epithelial spheres, budding off from the rostral end of each of the two plates of paraxial mesoderm (the segmental plates). Later, the ventromedial edge of each somite loses its epithelial character and becomes sclerotome, while the dorsolateral portion, the dermomyotome, remains epithelial for longer (see Bellairs, 1979, for review). The sclerotomes later give rise to the axial skeleton, while the dermis of the trunk and all the skeletal muscles arise from the dermomyotomes.

In order to explain the control of somite number in *Xenopus*, Cooke & Zeeman (1976) proposed a 'clock and wavefront' model, suggesting that a 'kinematic wave' precedes segmentation, which acts with some 'biochemical clock' to gate presumptive somite cells into groups for segmentation. The results of heat-shock experiments on amphibian (e.g. Elsdale *et al.* 1976; Cooke, 1978; Elsdale & Davidson, 1986) and chick (Veini & Bellairs, 1986) embryos have been taken as support for this model. For example, in *Xenopus* or *Rana*, a brief heat shock later causes a visible focal disruption of segmentation. The time interval between the shock and its visible effect is held to reflect the time interval between the commitment of any one group of cells to segment and the event of segmentation itself. The experiments were interpreted on the basis of the assumption that the shock perturbs a synchrony between the 'wave of determination' and the 'cellular clock'. This should cause a single visible segmental anomaly, appearing after a specific time interval equal to that between commitment to, and manifestation of, the act of segmentation.

By definition (see Slack, 1983), determination is a single event at which a cell becomes irreversibly committed to a particular fate. Heat-shock experiments of this kind are usually designed to answer the question: when does determination occur? The assumption is that in a continuous developmental process, only those cells undergoing the determinative event at the time of the shock will be sensitive to the disturbance and a localized change of fate will be observed. The position of the resulting anomaly should, therefore, reflect the time at which critical developmental decisions are made. To test this assumption, we have subjected 2-day-old chick embryos *in ovo* to transient heat shock; we have examined the

embryos, after further incubation for various periods, for the presence and position of segmental anomalies.

Materials and methods

Hens' eggs were incubated at 38°C for two days (stages 8–13; Hamburger & Hamilton, 1951). A window was made in the shell over the blastoderm, a few μl of Indian ink (10%, in Pannett–Compton's saline) was injected beneath the blastoderm to improve contrast between the embryo and the yolk, and the somite number recorded. The embryos were then subjected to heat shock.

The shell was sealed with tape and the eggs were placed in an incubator set to 55°C for 52 min, after which they were incubated at 38°C for the following periods: 1 day, 2 days, 3–5 days and 7 days (see below). Control embryos were treated in exactly the same manner except that they were exposed to 38°C throughout the experiment.

Most of the embryos incubated for 1 day post-heat shock were pinned out on Sylgard dishes, fixed in buffered formol saline, dehydrated in an alcohol series and stained as whole mounts with Fast Green made up in 100% ethanol. The somite number was recorded, and the embryos examined to determine the presence and position of somite anomalies.

Some of the embryos that exhibited distinct segmental anomalies after 1 day of post-heat-shock incubation were incubated for a further 24 h (until stage 16–21; Hamburger & Hamilton, 1951). These embryos were then pinned out on Sylgard dishes, bisected along the midsagittal plane and stained directly in a solution containing ZnI_2, OsO_4 and KI_3 at 55°C for 100 min (Keynes & Stern, 1984), washed in distilled water, dehydrated in a graded alcohol series, cleared in xylene and whole-mounted in Canada Balsam. Specimens were scored for abnormalities of the position of the spinal roots; these were compared to the positions of previously observed somite anomalies.

Some of the embryos that exhibited distinct segmental anomalies 1 day after heat shock were incubated for a further 4–6 days. They were then pinned out on Sylgard dishes, fixed in buffered formol saline, placed in 5% sucrose in phosphate-buffered saline (PBS, pH 7·4) for 24 h, then in 15% sucrose in PBS for 24 h, and then embedded in 7·5% gelatin (Sigma, 300 bloom) containing 15% sucrose in PBS. The specimens were then frozen and sectioned in a cryostat at 10 μm in either sagittal or coronal planes. After staining with haematoxylin, sections were scored for abnormalities of the development of vertebral cartilages; the positions of such abnormalities were scored and compared to those of previously observed somite anomalies.

Embryos incubated for 7 days after heat shock were processed to visualize the skeletal elements. They were fixed in 95% ethanol for 1 week, placed in acetone for 3 days, and then the skin and viscera were removed. They were then stained for 3 days in a solution containing Alcian Blue, Alizarin Red, acetic acid, and 70% ethanol (McLeod, 1980). After staining, the specimens were washed in distilled water and cleared through a graded series of KOH and glycerin solutions (1% KOH, followed by 20%, 50%, and 80% glycerin in 1% KOH; McLeod, 1980) over 4 weeks. The positions of abnormalities of the axial skeleton were then recorded.

Results

106 of the 273 (39%) embryos treated with heat shock exhibited discrete somite anomalies, which were observed after 24–48 h post-treatment incubation at 38°C. The anomalies consisted of either one small (16%) or one large (13%) somite, or two consecutive somites apparently fused together (71%). These anomalies appeared 6–7 somites (range: 5–8) after that last formed at the time of treatment. In some cases, a second and/or third anomaly was observed: in these embryos, the anomalies were separated from each other by an interval of 6–7 somites (range: 5–8) or a multiple of this distance (Fig. 1). Of the 106 embryos showing discrete anomalies, 90 (85%) exhibited anomalies on one side of the embryo only, while 16 (15%) exhibited anomalies on both sides; of this latter group, 8 embryos had bilateral anomalies (at the same rostral–caudal position on both sides of the embryo).

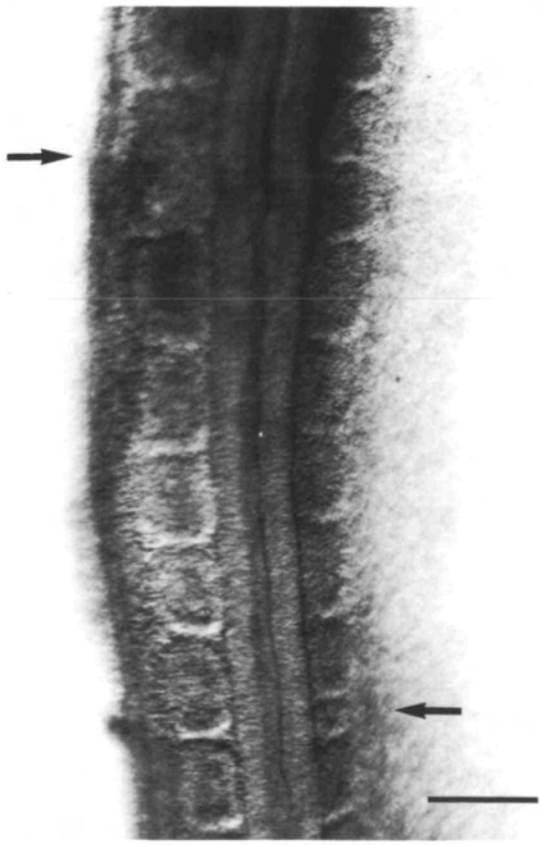

Fig. 1. Treatment-induced somite anomalies. Embryo heat shocked at the 7-somite stage; two anomalies can be seen (arrows): a fusion of somites 21–22 and a small somite at position 28. Rostral towards the top of the photograph. Bar, 100 μm.

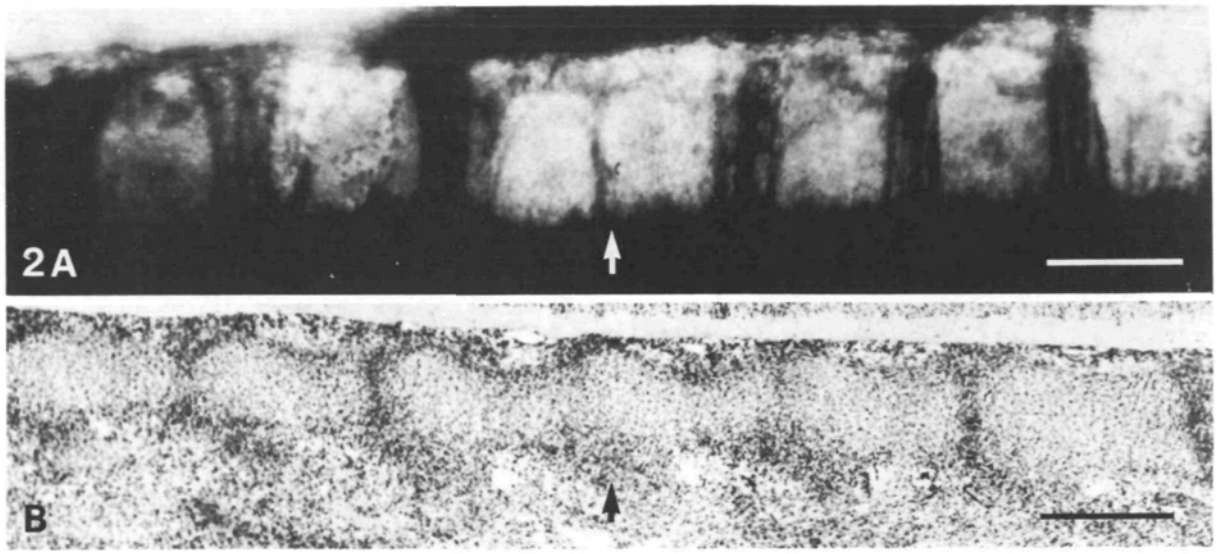

Fig. 2. Embryos heat shocked at the 10-somite stage. (A) Stained with ZnI_2/OsO_4 at stage 19 to show the spinal nerves: an anomaly can be seen between two consecutive nerve roots, preceded and followed by three normal segments. Rostral to the right of the photograph. Bar, 100 μm. (B) Fixed 3 days after heat shock, wax embedded, sectioned sagitally at 10 μm and stained with haematoxylin to show prevertebral condensations: the condensations at segmental levels 16–18 are fused (arrow). Rostral to the right of the photograph. Bar, 200 μm.

Of the 167 (61%) embryos not showing discrete somite anomalies, 121 (72%) were found to be normal, 16 (10%) were dead and 10 (6%) were severely affected. The latter category included embryos exhibiting malformed heads, tissue necrosis, neural tube defects and other generalized defects. Embryos that survived heat shock greater or longer than 52°C for 55 min showed nondiscrete somite anomalies which comprised many adjacent segments.

Some heat-shocked embryos showing discrete anomalies 1 day after treatment were subsequently stained with ZnI_2/OsO_4 on day 4 of development. Of these, 6 of 15 (40%) were found to have discrete anomalies of the spinal roots at the affected segmental level. In segments smaller than normal, the spinal nerve occupied the entire extent of the sclerotome. In large or fused segments, the spinal nerve was confined to the most rostral portion of the segment, while a much larger than normal caudal portion was devoid of axons (Fig. 2A).

6 of the 12 (50%) heat-shocked embryos that showed discrete somite anomalies one day after treatment, when examined after 2–4 days' further incubation at 38°C, exhibited discrete abnormalities in the condensation of sclerotome at the level of the affected somite. These anomalies consisted of two or three fused consecutive condensations (Fig. 2B).

16 of the 51 (31%) embryos that had been heat shocked on the second day of development and incubated at 38°C for 7 days after shock exhibited rib and vertebral anomalies. These were predominantly at a position corresponding to 6–7 segments after that last formed at the time of shock, and at 6–7 vertebral intervals thereafter. Anomalies were observed at a maximum distance of 26 segments (4×6·5 intervals) after that last formed at the time of heat shock (Fig. 3). The anomalies consisted of vertebrae exhibiting either two or three fused consecutive neural arches or ribs, a bifurcated rib, or an ectopic rib on the first lumbar vertebra (Fig. 3).

The frequency and position of anomalies observed after heat shock in somites and vertebrae is shown in Fig. 4 (note: segment 0 represents the last somite formed at the time of treatment in all embryos); these histograms show that a single heat shock, given to 2-day-old chick embryos, can generate multiple but discrete somite and skeletal anomalies, the first anomaly appearing about 6–7 segments after the last somite formed at the time of treatment. The anomalies are separated by a distance of about 6–7 segments, or a multiple of this distance, from each other (see Fig. 5).

Fig. 6 shows the number of vertebrae seen in the cervical and thoracic regions of the vertebral column of control embryos ($n = 6$) and heat-shocked embryos that exhibited vertebral anomalies ($n = 16$): 5 of the heat-shocked embryos showed normal numbers of cervical and thoracic segments, while 11 showed variations in the number of segments within these regions.

Control embryos

No anomalies were observed in any of the control ($n = 120$) embryos.

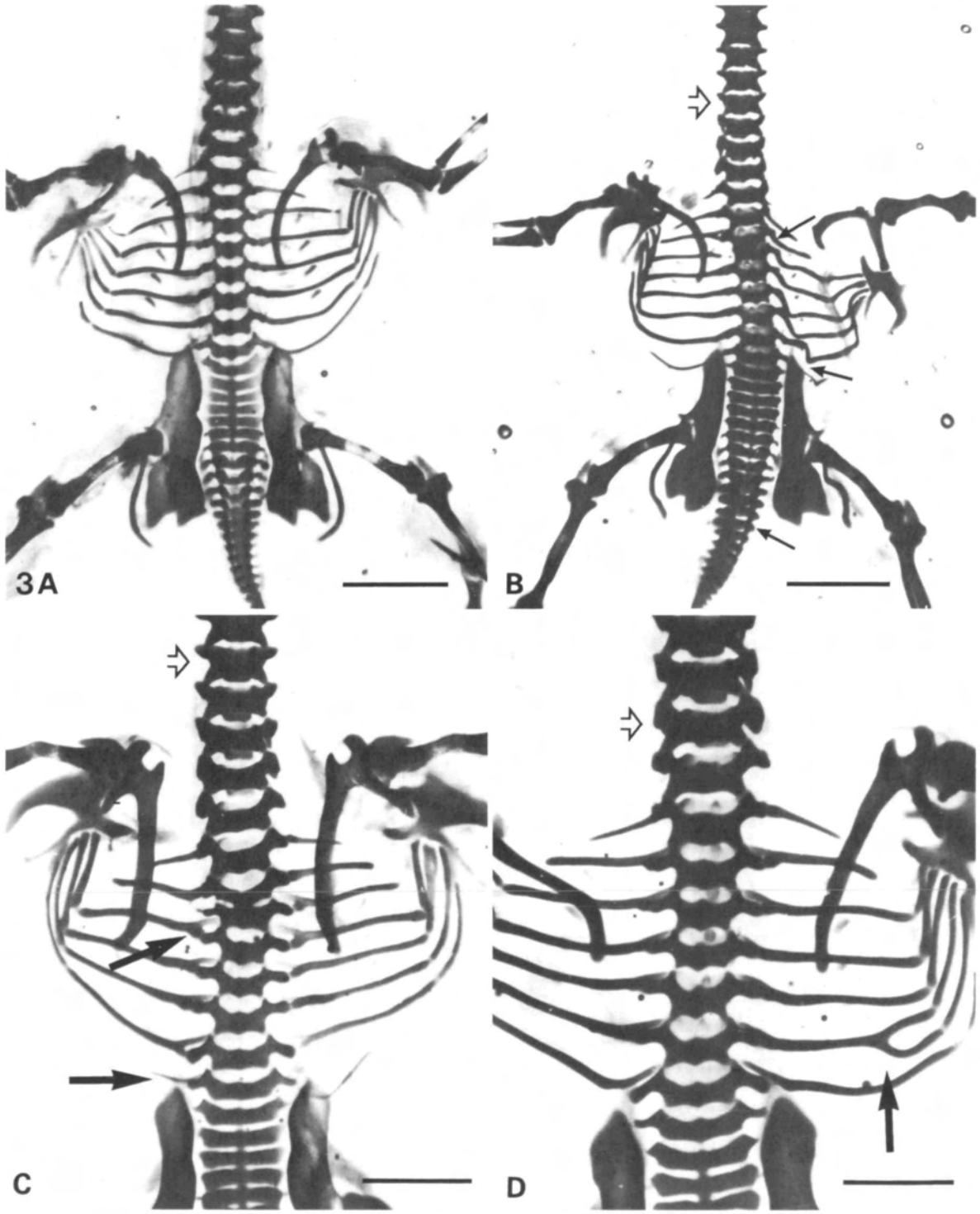

Fig. 3. Embryos heat shocked on the second day of development, fixed and stained as a whole mount with Alcian Blue 7 days after heat shock to show cartilaginous vertebrae. (A) Control embryo. (B) Three anomalies are visible (solid arrows): a vertebral fusion with an associated detached rib at cervical level 16 to thoracic level 1 (C16–T1), an ectopic lumbar rib at L1, and the third caudal vertebra has a malformed neural arch. The ribs on the right side of the embryo appear distorted due to the position of the embryo when pinned, and are not anomalous. (C) Two anomalies are visible (arrows): a vertebral fusion at C16–T1 and a malformed rib at T5. (D) A bifurcated rib is visible at T4 (arrow). In each case, the last segment formed at the time of treatment is shown by an open arrow. [Note: normal vertebral composition of adult fowl: 16 cervical (last two usually with ribs), 5 thoracic (all with ribs), 4–5 lumbar, 5 sarcal, 6 caudal, 6 coccygeal and a few terminal, fused, vertebrae (pygostyle)]. Bars, 5 mm in A and B, 3 mm in C and D.

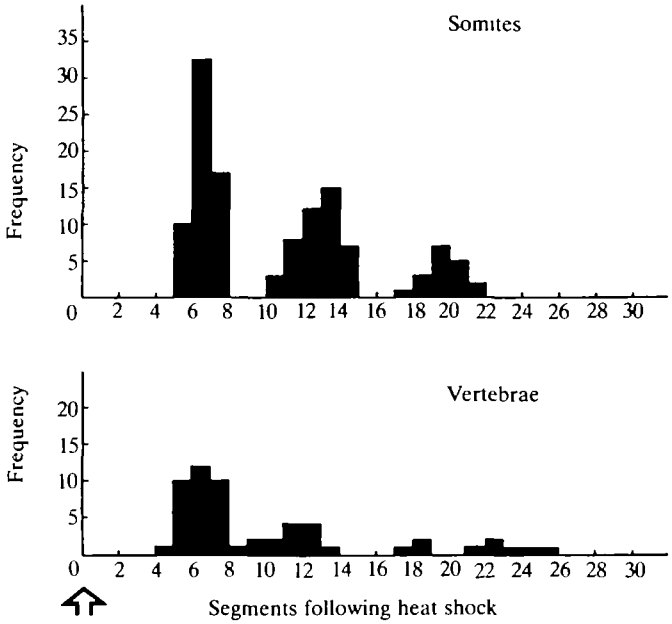

Fig. 4. Frequency histograms showing the occurrence and position of anomalies observed after heat shock in A, somites and B, vertebrae, The y axis shows the total number of cases of a given segment being anomalous, the position of that segment (x axis) being measured relative to the time of the shock (arrow).

Discussion

Our results show that a single episode of heat shock given to 2-day-old chick embryos can generate multiple but discrete somite and vertebral anomalies. Each vertebra can be correlated with a specific somite (Muller & O'Rahilly, 1986), allowing for the fact that the most rostral somites in the chick embryo give rise to occipital structures (Noden, 1983; Lim *et al.* 1987). The distance between the last somite formed at the time of treatment and the first affected segment (somite or vertebra) is 6–7 segments, or a multiple of this distance. Each anomaly is restricted to one, or at most two, contiguous segments. Embryos may display one or more anomalies, separated from each other by 6–7 segments, or a multiple of this distance. When such embryos are stained to visualize the pattern of spinal nerves, it is seen that the periodic arrangement of nerves is also abnormal at positions corresponding to those of the affected segments.

Our experiments allow us to address a number of related questions regarding the control of segmental pattern in the chick embryo. First, we can ask whether Cooke & Zeeman's (1976) 'clock-and-wavefront' model, or Slack's (1983) 'clock-and-gradient' modification of this model, is valid for chick embryos. Second, we can address whether somite number and size are controlled in amniotes and, if so, how. Finally, we can consider whether such experiments can establish the time at which determination occurs.

Is the clock-and-wavefront model valid?

If the results of the heat-shock experiments of Elsdale, Cooke and co-workers (e.g. Cooke, 1978; Elsdale *et al.* 1976; Elsdale & Pearson, 1979; Elsdale & Davidson, 1986) are to be taken as evidence in favour of a clock-and-wavefront (Cooke & Zeeman, 1976) or clock-and-gradient (Slack, 1983) model, it is important to realize that both of these models propose a *single* event combined with a cyclic one. The single event may be associated with the passage, once-and-for-all, of a wavefront, or it may be a standing gradient with thresholds of 'interpretation'. Our results support the idea of some cyclic mechanism, but are not consistent with the notion of a single event that gates cells for segmentation.

The evidence for a 'clock'

The finding that a single disturbance, such as brief heat shock, can generate multiple anomalies separated from each other by a constant distance suggests that some cyclic event is involved in segmentation. What might be the cellular basis for this cyclic event?

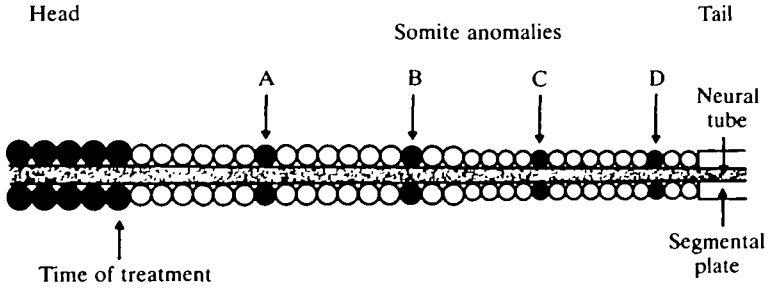

Fig. 5. Summary, in 'cartoon' form, of the mean positions of anomalies seen after a single heat shock, relative to the position of the last somite formed at the time of treatment. The first anomaly (A) appears about 6–7 segments after the last somite formed at the time of treatment. Each anomaly is separated from the previous by a distance of 6–7 somites (or vertebrae), or a multiple of this distance. It should be noted that the diagram is an exaggeration of the results in that it represents a composite of all the experimental embryos in this paper (cf. data in Fig. 4A and B), and therefore no single embryo ever showed the pattern illustrated. Only a few of the anomalies observed were bilateral.

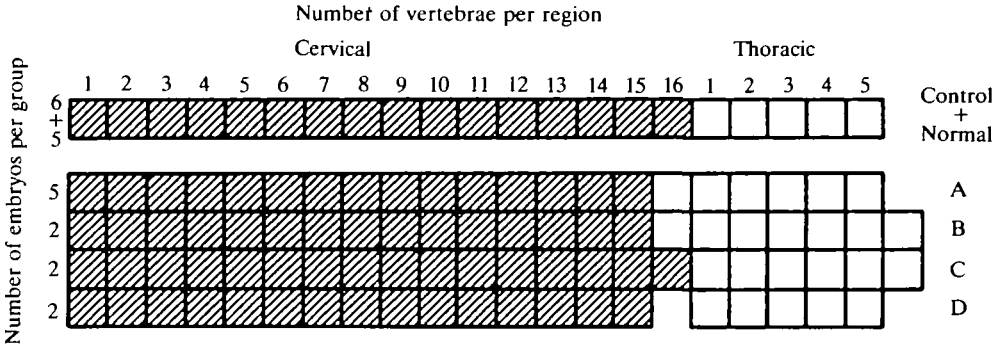

Fig. 6. Diagram showing the number of cervical and thoracic vertebrae in embryos heat shocked on the second day of development, assessed 7 days after treatment. The upper block diagram represents the normal arrangement, seen in six untreated controls and in five of the heat-shocked embryos. The lower panels (A–D) show abnormal patterns seen only in treated embryos. (A) The 16th cervical vertebra (C16) had a thoracic-like pair of ribs in five embryos. (B) C16 had a thoracic-like rib and there was a lumbar rib at L1 (two embryos). (C) In two embryos, L1 had a pair of ribs. (D) Two embryos only had 15 cervical vertebrae.

In the brachial region of the chick embryo, one pair of somites forms approximately every 1·5 h (Menkes *et al.* 1961). A distance of 6–7 somites is therefore equivalent to a time interval of about 9–10 h. Our results suggest that there might be a clock-like event that is involved in gating those cells that will segment together. One possibility would be that this event is related to the cell division cycle. If this is the case, we would predict the length of the cell division cycle of these cells to be of the order of 9–10 h.

Moreover, if the distance between anomalies is dependent upon the length of the cell cycle, we would expect some degree of cell division synchrony between those cells that segment together. This appears to be the case: Stern & Bellairs (1984) showed that a high mitotic index is often found at or near the rostral end of the segmental plate.

The evidence against a 'wave' or 'gradient'

We have observed that a single heat shock can cause multiple anomalies separated from each other by a constant distance. It is perhaps interesting that other workers have in fact observed multiple anomalies in response to a single insult: in early gastrula amphibian embryos, heat shock (Elsdale *et al.* 1976; Cooke, 1978) or brief exposure to nocodazole (Elsdale & Davidson, 1986) both result in multiple somite anomalies. The occurrence of these multiple anomalies is not compatible with the existence of either a single propagating wave or a gradient that gates the clock.

Is the total number of somites controlled in amniotes?

This is a difficult question to answer definitively, because most investigators have looked at their experimental embryos *during* somite formation, rather than after completion of the process (e.g. Elsdale *et al.* 1976; Veini & Bellairs, 1986). Therefore, these investigators may have been studying the rate of somite formation, rather than the control of somite number. One way to examine whether or not embryos are able to control the total number of somites formed is to investigate whether embryos smaller or larger than normal are able to produce the normal number of somites.

Cooke (1975) stated that '*when overall cell number of early vertebrate embryos is reduced, cell numbers developing along each pathway are reduced to give a normally proportioned whole-body pattern*'. In his experiments involving surgical removal (Cooke, 1975) or addition (Cooke, 1978) of blastula cells, he observed that *Xenopus* embryos appear to maintain somite number at the expense of somite size and concluded that vertebrate embryos regulate somite number (see Cooke, 1978 for review). He felt that '*the observed constancy and regularity of element size and number is embarrassing for all known prepattern models*' (Cooke, 1975).

Experiments in which embryonic size is altered experimentally, and the final number of vertebrae assessed, have been performed only rarely in amniotes. One example is the experiment of Gregg & Snow (1983), which is somewhat analogous to the removal of cells from the blastula (Cooke, 1975): they treated early-somite-stage mouse embryos with sufficient mitomycin C to kill up to 80 % of cells in the blastoderm (see Snow & Tam, 1979) but later were unable to demonstrate regulation of vertebral number.

Other authors have shown that the final number of segments can be altered by rearing at abnormal temperature. This is the case in embryos of all vertebrate classes (reviewed by Fowler, 1970). Somite-stage embryos of bony fish (e.g. Orska, 1962), amphibians (e.g. Lindsey, 1966), reptiles (e.g. Fox, 1948), birds (e.g. Lindsey & Moodie, 1967) and mammals (e.g. Lecyk, 1969) exposed to altered

temperature exhibit an altered number of vertebrae relative to parents and/or untreated siblings. Such changes have been correlated with prior changes in somite number (Orska, 1962). These findings do not support Cooke & Zeeman's (1976) generalization that '*somite number is highly constant across a wide range of developmental temperatures*'. It therefore appears likely that most vertebrate embryos are unable to control the total number of somites.

Can heat-shock experiments address when determination occurs?

Among the decisions made by cells during the process of segmentation, the following can be distinguished: [1] whether to become somitic (rather than another mesodermal derivative); [2] whether to become dermomyotome or sclerotome (myogenic, dermatogenic or chondrogenic); [3] when to segment; [4] whether to become cervical, thoracic, etc. and [5] whether to become rostral or caudal (if sclerotome). The experiments described in this paper could be used to address the last three decision-making processes, albeit in an indirect way.

Control of somite size and the timing of segmentation

The size of a somite must depend, at least to some extent, on the number of cells that segment together (see Bellairs, 1979). It is unlikely, however, that the number of cells destined to form each somite is allocated by a cell-counting process, because the normal-sized somites seen in haploid embryos (which have smaller cells) contain twice the normal number of cells (Hamilton, 1969). Instead, it seems more likely that the size of each somite is related directly to some earlier event, which designates those cells that will segment at the same time. Moreover, the addition or removal of paraxial mesoderm in chick embryos does result in a change in somite size (Menkes & Sandor, 1977), which suggests that each cell is committed to segment at a particular time.

Our results indicate that heat-shocked chick embryos show alterations in somite size, which could reflect an incorrect number of cells being incorporated into the affected somites. These considerations imply that heat shock could affect the process by which cells become programmed to segment at a particular time. This possibility will be investigated in a later publication.

Regional specification

Can the present experiments help us to ascertain when the cells of different somitic regions become determined as members of any specific axial region? The methods used in this study have allowed us to analyse embryos in terms of the number of vertebrae in each region. The results show that heat shock can produce variations in the number of vertebrae in each region of the vertebral column studied. These variations could result either from deletion of a segment or from a change in the position of the boundary between adjacent regions. It is possible that the mechanisms that confer regional characteristics to particular somite derivatives are linked to the processes that program the cells of that segmental level to segment at a particular time.

Rostrocaudal determination

Motor axon outgrowth (Keynes & Stern, 1984) and neural crest cell migration (Rickmann *et al.* 1985) from the developing neural tube occur only through the rostral half of each sclerotome. This selectivity is due to differences between the rostral and caudal sclerotome rather than to intrinsic segmentation in the neural tube (Keynes & Stern, 1984; Stern & Bronner-Fraser, in preparation). When does a presumptive sclerotome cell become determined as rostral or caudal? Several considerations led us to propose that rostrocaudal commitment occurs during the formation of a somite (Stern & Keynes, 1986, 1987). If this is the case, it may be relevant to remember that rostral cells lie adjacent to an intersomite border for a longer period of time than do caudal cells. Commitment to one or the other half could be linked to this time difference.

In the present experiments, we found that in abnormally large somites only the caudal part was enlarged; abnormally large rostral parts were never observed. We believe that this is significant. It suggests that the number of cells specified as rostral is a function of the number of cells facing an existing border (at the rostral end of the segmental plate), which in turn depends on the *geometry* (i.e. the mediolateral width) of the segmental plate. The number of cells specified as caudal, on the other hand, may be a function of the *number of cells* destined to condense together into the same somite. Heat shock does not appear to affect the width of the segmental plate, and therefore would be unlikely to alter the size of the rostral half of the sclerotome. It could, however, affect the number of cells that will condense together, and thus the extent of the caudal half. We take this as indirect evidence that rostrocaudal determination occurs during somite formation.

Conclusions

The results presented in this paper provide evidence that a cyclic event is involved in the allocation of cell populations destined to segment together to form individual somites in the chick embryo. We suggest

that this cyclic event may be linked to the cell division cycle. However, our results do not support the notion that a 'standing gradient' or a 'propagating wave' gates this event. The findings argue against the idea that there is a single determinative step at which cells become committed to form individual segments.

Heat shock also affects the size and number of segments, as well as the relative size of the rostral and caudal sclerotome halves. We suggest that heat shock primarily affects the number of cells that segment together and that this is responsible for all the observed effects.

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