Periodic segmental anomalies induced by heat shock in the chick embryo are associated with the cell cycle

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Summary

This study provides evidence that cells destined to segment together into somites have a degree of cell division synchrony. We have measured the duration of the cell division cycle in somite and segmental plate cells of the chick embryo as 9·5 h using [³H]thymidine pulse-and-chase. Treatment of embryos with any of a variety of inhibitors known to affect the cell division cycle causes discrete periodic segmental anomalies: these anomalies appear about 6–7 somites after treatment and, in some cases, a second anomaly is observed 6 to 7 somites after the first. Since somites take 1·5 h to form, the 6- to 7-somite interval corresponds to about 9–10 h, which is the duration of the cell cycle as determined in these experiments. The anomalies are similar to those seen after heat shock of 2-day chick embryos. Heat shock and some of the other treatments induce the expression of heat-shock proteins (hsp); however, since neither the expression nor the distribution of these proteins relate to the presence or distribution of anomalies seen, we conclude that hsps are not responsible for the pattern of segmental anomalies observed. The production of periodic segmental anomalies appears to be linked to the cell cycle. A simple model is proposed, in which we suggest that the cell division cycle is involved directly in gating cells that will segment together.

Key words: segmentation, heat-shock proteins, cell division cycle, somites, chick embryo, mesoderm, pattern formation.

Introduction

We have shown recently that a single brief treatment with heat shock produces up to four discrete segmental anomalies in 2-day chick embryos. The first anomaly appears 6–7 somites after that last formed at the time of treatment, and the remainder appear at 6- to 7-somite intervals thereafter (Primmett et al. 1988). We argued that the periodicity of these anomalies reflects some underlying cyclic event. In the chick embryo, one somite takes about 1·5 h to form (Menkes et al. 1961); 6–7 somites therefore correspond to an interval of 9–10 h. If the postulated cyclic event is the cell division cycle, then the duration of the cell cycle of cells constituting chick segmental plates and somites should also be 9–10 h.

However, it is possible to explain the pattern seen after heat-shock treatment of chick embryos in another way. It has been reported by workers studying a variety of developing systems that the heat-shock proteins (hsp) produced in response to heat shock can lead to defects in body pattern directly (Mitchell & Lipps, 1978; German, 1984; Veini & Bellairs, 1986; Mirkes, 1987). Therefore, it is conceivable that the expression

of these proteins after heat shock in the chick is spatially periodic. The experiments described in this paper are designed to establish whether the segmentation anomalies observed are caused by the production of heat-shock proteins, or by interference with the cell division cycle.

Materials and methods

[³H]Thymidine pulse-and-chase

Chick embryos of 2 days' incubation were removed from the eggs and placed into 5 μCi [³H]thymidine (³H-TdR; specific activity: 851 GBq mmol⁻¹, Amersham) in 0·5 ml of Pannett & Compton saline for 45 min (= 'pulse'). The embryos were then washed five times in fresh saline and cultured at 37°C on a vitelline membrane over a pool of thin egg albumen containing 5 μM-'cold' thymidine (= 'chase'). They were fixed in absolute ethanol at 4°C, one embryo per hour, for the following 20 h. The fixed embryos were stained lightly with Fast Green in absolute alcohol and cleared and stored in oil of cedarwood. They were then embedded in paraffin wax, sectioned in the sagittal or coronal plane at 10 μm and mounted on glass slides using gelatin/albumen as adhesive. The slides were dewaxed in xylene and hydrated in a series of

alcohols, and then dipped while still wet into molten Ilford K2 emulsion diluted 1:1·5 with water containing 0·1 % glycerol. They were exposed for 4 weeks at 4°C and developed in Kodak D19. After fixation, the slides were thoroughly washed in running tap water, then placed in citrate/phosphate buffer (0·1 M-citric acid, 0·2 M-Na₂HPO₄, pH 4·1) for 5 min and the sections stained with 20 μM-acridine orange made in the same buffer.

Sections showing both somites and segmental plate from each embryo were observed using an Olympus Vanox–T microscope using an eyepiece graticule and a ×40 oil immersion, fluorescence-free objective. The total number of cells, of labelled cells and the number of labelled and unlabelled mitoses were scored in each field. The graticule was a square, large enough to accommodate the diameter of a somite (100 μm). Field positions were assigned relative to the rostral end of the segmental plate: the cleft between this end of the plate and the most caudal somite was designated as '0', fields rostral to this point were designated as positive and field positions within the segmental plate were assigned negative values. From the parameters measured in each field, values for mitotic index (MI), labelling index (LI) and the proportion of mitoses that were labelled (percent labelled mitoses; PLM) were calculated:

$$MI = \frac{\text{number of mitoses/field}}{\text{total no. cells/field}}$$

$$LI = \frac{\text{no. labelled cells/field}}{\text{total no. cells/field}}$$

$$PLM = \frac{\text{no. labelled mitoses/field}}{\text{total no. mitoses/field}}$$

The data obtained for PLM were analysed by Dr G. G. Steel, using computer-assisted curve-fitting methods (Steel & Hanes, 1971); the resulting 'best fit' yielded optimized parameters for cell cycle duration and transit times for G_2, S and G_1 phases of the cell cycle.

Chemical treatments

Hens' eggs were incubated at 38°C to about stage 13 (Hamburger & Hamilton, 1951), a window was made in the shell over the embryo and a few microlitres of a solution of Indian ink in calcium-/magnesium-free saline (CMF) were injected immediately under the embryo to improve contrast between the embryo and yolk. The somite number was recorded and the eggs were then subjected to one of the following treatments: 100 μl of a stock solution of either L- or D-phenylalanine (L-Phe or D-Phe [Sigma]; 5 mg ml⁻¹; Palén & Thörneby, 1981), bromodeoxyuridine (BUdR [BDH]; 0·5 μg ml⁻¹; Freeman, 1985), hydroxyurea ([BDH]; 10 mg ml⁻¹; Herken, 1980), mitomycin C ([BDH]; 0·5 mg ml⁻¹; Murphy, 1960), 5-fluorouracil ([Sigma]; 3 μg ml⁻¹; Murphy, 1960), colchicine ([Sigma]; 0·4 mg ml⁻¹; Stern, 1979), or nocodazole ([a gift from Dr G. Ireland]; 0·5 mg ml⁻¹; Elsdale & Davidson, 1986) in CMF were injected immediately beneath the embryo. The shell was then sealed with tape and the eggs incubated for the following periods: (1) embryos subjected to L- or D-Phe were incubated *in ovo* at 37°C for a further 26 h; (2) embryos subjected to all other treatments were incubated for 2 h *in ovo* at 37°C, explanted into New (1955) culture, washed five times with Pannett–Compton saline and then cultured for 24 h at 37°C. Subsequent to these treatments, all embryos were fixed in neutral buffered formol saline, dehydrated in an alcohol series, stained with Fast Green FGF (BDH) in 100 % ethanol, and cleared and stored as whole mounts in cedarwood oil. The somite number was recorded and the embryos scored for the presence and position of somite anomalies. Control embryos

were treated in exactly the same manner except that they were injected with CMF only.

BUdR treatment and immunohistochemistry

As cells incorporate BUdR into DNA only during S-phase (Stockwell, 1979), brief treatment with this agent, followed by immunocytochemical detection of the cells that have incorporated it with an antibody (Dolbeare *et al.* 1983) can be used to estimate the proportion and spatial distribution of cells in S-phase.

A modification of the method described by Dolbeare *et al.* (1983) was used. 100 μl of a stock solution of BUdR (0·5 μg ml⁻¹) in CMF were injected immediately beneath the embryo, after which the shell was sealed with adhesive tape and the eggs incubated for a further 2 h at 37°C. The embryos were then removed from their eggs, washed five times with Pannett–Compton saline and then either fixed immediately in cold (4°C) 100 % ethanol for 15 min, or explanted into New (1955) culture and then fixed after a further 24 h incubation at 37°C. The fixed embryos were transferred to 5 % sucrose in PBS for a few hours, then to 15 % sucrose in PBS overnight and finally embedded in 7·5 % gelatin (Sigma, 300 bloom) in 15 % sucrose in PBS. The blocks were sectioned in a cryostat at 10 μm in the coronal plane.

Appropriate sections were hydrolysed in 1·5 N-HCl at room temperature for 20 min and washed with PBS, after which a solution containing a 1:10 dilution of anti-BUdR antibody (Becton Dickinson), 0·5 % bovine serum albumin (BSA) and 0·05 % Tween-20 in PBS was laid over the sections for 1 h at room temperature; the slides were washed with PBS, stained using either fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat antimouse IgG (1:50 in PBS with 0·05 % Tween 20 and 0·5 % BSA) for 1 h, and washed again with PBS. The sections were then viewed with fluorescence optics and examined for regional variations in the proportion of fluorescent cells in somites and segmental plate.

Immunohistochemical localization of heat-shock proteins

The shell was sealed with tape and the eggs were subjected to heat shock (55°C for 52 min; Primmett *et al.* 1988), after which they were incubated at 38°C for 0, 1, 3, 9 or 24 h. Embryos were then removed from the eggs, fixed in buffered formol saline for 20 min, scored for the presence and position of somite anomalies, transferred to 5 % sucrose in PBS at 4°C for 12 h, then to 15 % sucrose in PBS at 4°C for 12 h and then embedded in gelatin containing 15 % sucrose. They were sectioned coronally at 10 μm with a cryostat, and the sections air dried and stored at 4°C. Control embryos were processed in exactly the same manner, but were incubated at 38°C only.

The sections were rinsed for 30 min in PBS at 38°C to remove the gelatin, exposed to 0·1 % Triton X100 in PBS for 1 min to permeabilize the cells, washed in PBS and then blocked for 20 min in 0·3 % BSA in PBS. They were then incubated for 1 h in rabbit anti-chick hsp 24 (1:20), hsp 70 (1:50), or hsp 89 (1:50) antiserum (Collier & Schlesinger, 1986) in PBS containing 0·5 % BSA and 0·05 % Tween-20. The sections were then washed with PBS and incubated for 1 h in peroxidase-conjugated goat anti-rabbit IgG (Sigma; 1:50 in PBS with 0·5 % BSA and 0·05 % Tween 20). Peroxidase activity was visualized using diaminobenzidine (Aldrich) in 0·1 M-Tris buffer (pH 7·6) as the substrate.

Characterization of heat-shock proteins

The embryos were subjected to L- or D-Phe, heat shock, BUdR (as described above) or New (1955) culture. They were

then removed from the eggs or culture dishes, and pinned out in Sylgard (Dow Corning) dishes containing 0·1 % EDTA in CMF at 37 °C to facilitate the dissection of paraxial mesoderm and somites. This tissue was collected in Eppendorf tubes containing 200 μl of 1 mM-phenylmethylsulphonylfluoride (PMSF) in CMF at 4 °C. Tubes were then centrifuged for 3 min at 100g at 4 °C. The supernatant was removed, the pellets resuspended in 1 ml of CMF with 1 mM-PMSF at 4 °C and the tubes centrifuged again at 11 600g at 4 °C. The supernatants were quickly frozen by placing the tubes in dry ice and the samples stored frozen at −20 °C for a maximum of one week prior to protein assay.

Protein estimations were performed according to Bramhall *et al.* (1969): (1) after thawing the tissue samples, 150 μl of sample buffer (stock solution: 300 mM-Tris–HCl at pH 6·8, 10 % SDS, 50 % glycerol, and 0·05 % bromophenol blue; stock diluted 1:5) were added to each Eppendorf tube, the tubes placed in boiling water for 5 min, cooled to room temperature and then centrifuged for 3 min at 11 600g; (2) 3 μl of sample for each tube were applied to a 1 cm square of Whatman 50 hardened filter paper, which was then fixed in a 5:1:4 solution of methanol, acetic acid and deionized water, stained for 30 min in a 4:2:4 solution of methanol, acetic acid and deionized water containing 0·08 % Coomassie brilliant blue R250 (Sigma), rinsed in tap water, destained in 7·5 % methanol and 5 % acetic acid in deionized water, dried and then placed in an Eppendorf tube; (3) 1 ml of 66 % methanol/1 % ammonia was added to the tubes, which were then vortex mixed for 10 s; (4) 15 min later, the absorbance (590 nm) of the protein solutions was determined and read from a linear plot of concentration of standards; (5) finally, a sufficient volume of sample buffer was added to each tube to standardize the protein concentration of all samples.

Polyacrylamide minigels were prepared according to Matsudaira & Burgess (1978) using the Laemmli (1970) buffer system: (1) about 5 μg of protein were separated on 4–15 % gradient polyacrylamide SDS-gels (200 V, constant voltage) under reducing conditions (dithiothreitol); (2) the gels were then blotted electrophoretically onto Immobilon membranes for 1·25 h using Novablot semidry transfer buffer (48 mM-Tris, 39 mM-glycine, 20 % methanol, and 0·0375 % SDS) and the LKB Novablot multiphor system (transfer conditions: 0·8 mA cm^{-2} filter paper area); (3) the transfer membranes were stained with Ponceau S (BDH), washed free of the stain and then blocked overnight at 4 °C with 5 % BSA in Tris–saline (20 mM-Tris–HCl buffer, pH 8·2, containing 0·9 % NaCl, 5 % BSA and 20 mM-sodium azide).

At room temperature with constant mild agitation, the transfer membranes were washed three times for 5 min in 0·1 % BSA Tris–saline, incubated for 1 h in rabbit anti-chick hsp 24, hsp 70 or hsp 89 antisera diluted (1:200) in 0·1 % BSA Tris–saline. The membranes were washed three times for 5 min each in 0·1 % BSA Tris–saline and incubated for 1·5 h with gold-labelled goat anti-rabbit IgG (Janssen AuroProbe) diluted (1:100) in 0·1 % BSA Tris–saline containing 1:20 gelatin; washed thoroughly and silver enhanced for 5 min using the IntenSE II system. Finally, the membranes were washed for 5 min in deionized water and then dried between filter papers.

Results

[³H]Thymidine autoradiography

The percentage of labelled mitoses in the segmental plate and somites of 2- to 3-day chick embryos (17 embryos; 6 data points per embryo) after ³H-TdR
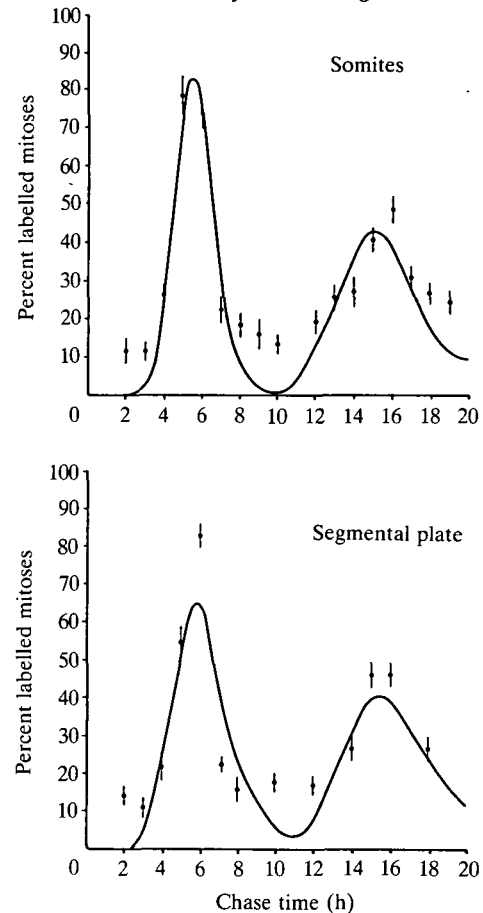

Fig. 1. Proportion (%) of labelled mitoses in cells within somites (top diagram) and presomitic paraxial mesenchyme (bottom) from 2-day chick embryos. The X-axis represents the length of chase in hours.

Table 1. *Optimized values for cell cycle duration* (T_C) *and transit times for* G_2 (T_{G2}), S (T_S), *and* G_1 (T_{G1}) *phases of the cell cycle (in hours) for cells occupying the segmental plate and somites in 2- to 3-day chick embryos*

Parameter	Mean	S.D.	Median
(a) Segmental plate			
T_C			9·5
T_{G2}	5·1	1·5	4·9
T_S	2·3	0·2	2·3
T_{G1}	2·3	0·6	2·2
(b) Somites			
T_C			9·5
T_{G2}	4·5	0·7	4·4
T_S	2·2	0·4	2·2
T_{G1}	3·3	2·3	2·7

pulse-and-chase is shown in Fig. 1. Optimized values for G_1, S and G_2 transit times and cell cycle durations are given in Table 1; median values obtained for cell cycle duration were identical in the segmental plate and somites, 9·5 h.

The autoradiographs obtained after ³H-TdR pulse-

2A

B
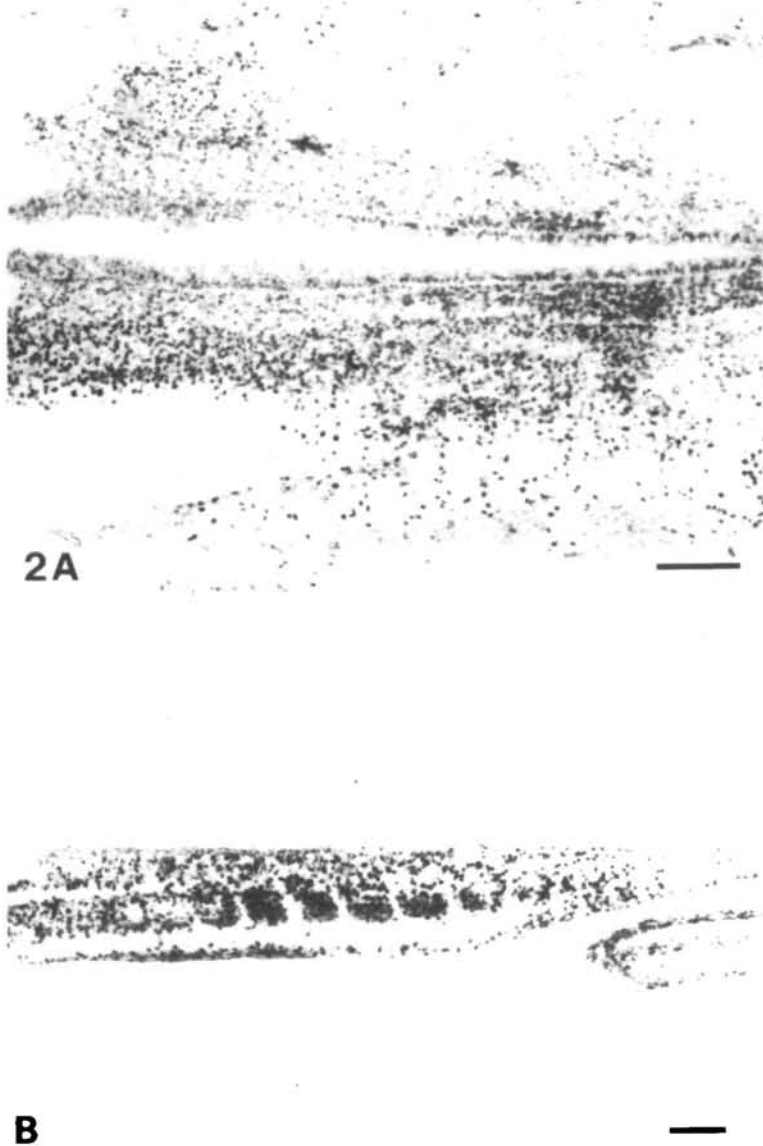
Fig. 2. Autoradiographs of ³H-TdR-labelled sections of 2-
day chick embryos. (A) In the segmental plate, a discrete
region of high labelling is seen towards the cranial end of
the plate (right in the photograph) and another, broader
region is seen at its caudal end (left). Coronal section. Bar,
220 μm. (B) In more cranial (somite) regions of another
embryo (sectioned sagittally), a region about two somites in
length displays an elevated labelling index. This region lies
about four somites rostral to the tip of the segmental plate.
Bar, 110 μm.

and-chase revealed the presence of discrete regions of
high labelling index separated from each other by
regions of lower labelling index in the segmental plate
(Fig. 2A). In most embryos (10/16), a discrete region of
high labelling index was seen near the tail end of the
segmental plate. In some embryos (7/17), there was
also a wider and more diffuse region of labelling in the
middle of the plate and, in a few (4/17), a concentrated
region of high labelling index was seen at its rostral end.

Table 2. *Effects of antimitotic agents on somite
formation in the chick embryo*

Treatment	Concentration (mg ml⁻¹)	n	% Anomalies
Colchicine	0·4	17	41
Nocodazole	0·5	17	24
Mitomycin C	0·5	16	25
5-fluorouracil	3·0	18	28
Bromodeoxyuridine	0·5	15	53
Hydroxyurea	10	13	46
L-Phenylalanine	5	19	38
D-Phenylalanine	5	19	0

See Fig. 4 for the position of the discrete segmental anomalies.

Three embryos displayed all three regions of high
labelling index within the segmental plate.

In the somites, similar discrete regions of high
labelling index were found, which were limited to one
or two consecutive segments. In a few (4/17) embryos,
a discrete region of high labelling index was seen in the
most caudal epithelial somite. Some (7/17) displayed a
discrete region of high labelling index about 4–6
somites rostral to the last somite (Fig. 2B), and others
(7/15) showed a similar peak of labelling index some
10–12 somites rostral to the last somite.

Fig. 3 is a three-dimensional graph illustrating the
passage of labelled cell populations through the differ-
ent stages of somitogenesis. It includes data from all
sections counted, summarized into three separate plots:
the left (black shading) represents a population of cells
labelled while it was at the caudal (tail) end of the
segmental plate, the middle plot (stippled) corresponds
to populations labelled while in the middle region of the
segmental plate, whereas the right (white) plot rep-
resents cells labelled at the rostral end of the plate. The
angle of each plot to the X-axis (field number) was
chosen in relation to the rate of somite formation (one
somite pair per 1·5 h; Menkes et al. 1961). It is found
from this figure that the interval between adjacent
peaks of labelling index corresponds to 6–7 segments,
regardless of the time at which the population of cells
became labelled. A similar result is obtained by plotting
mitotic index (MI) *versus* field number as shown in
Fig. 4: peaks of MI are found separated by regions of
lower MI in the segmental plate and somites. The
distance between adjacent peaks is of the order of 6–7
(range = 4–9) somite lengths in the segmental plate and
somites.

Cell cycle inhibitors

Embryos were treated with a variety of chemical agents
known to affect specific stages of the cell cycle. The
results are summarized in Table 2. All of the agents
used led to discrete somite anomalies, visible after 24 h
incubation following treatment (Fig. 5). The anomalies
consisted of either one small somite, one large somite,
or two consecutive somites fused together (Fig. 6) and
appeared about 6–7 segments (range = 6–8 for L-Phe,
colchicine and nocodazole; 5–8 for mitomycin C, 5-
fluorouracil, BUdR and hydroxyurea) after the last
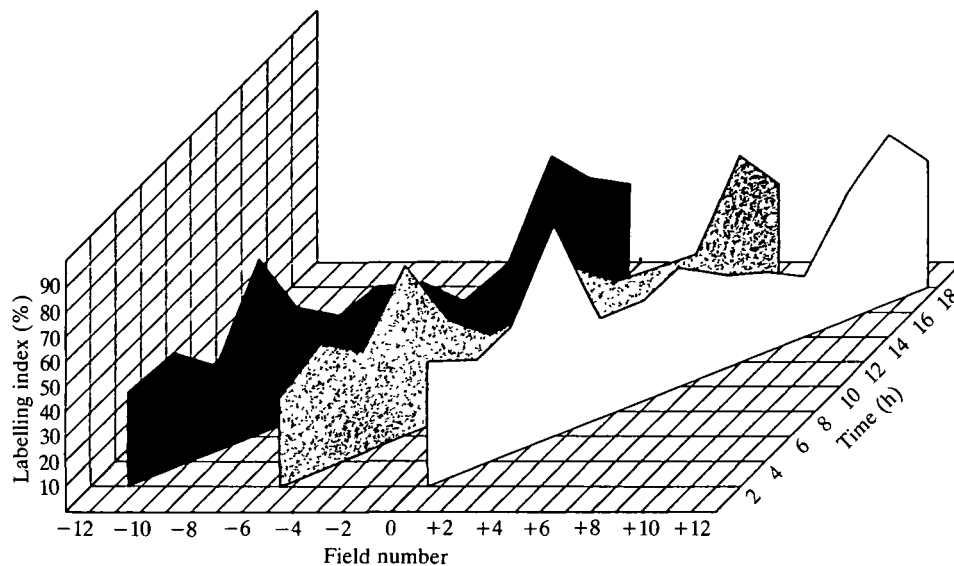
Fig. 3. Three-dimensional graph of labelling index (Y-axis) *vs* field number (X-axis) *vs* chase time (Z-axis). 1 field = the rostrocaudal length of 1 somite; field 0 corresponds to the rostral end of the segmental plate (positive fields correspond to somites, negative fields to segmental plate). Each of the three plots reflects the passage of a labelled cell population through the different stages of somitogenesis: since somites take 1·5 h to segment and 1 field equals one somite, cells at the caudal end of the segmental plate (at the -12th field; black) at T = 2 h will occupy the -1th field (i.e. the rostral end of the plate) at T = 18 h; similarly, cells at the -6th (stippled) and 0th (white) fields at T = 2 h will occupy the $+5^{th}$ and $+11^{th}$ fields at T = 18 h, respectively. Note that the interval between adjacent peaks on each of these plots equals 6–7 fields (= somites) on the X-axis and 9–10 h on the Z-axis.

somite formed at the time of treatment. A second anomaly, appearing 12–14 segments after treatment, was observed in 5 cases, in embryos treated with L-Phe, colchicine, nocodazole, 5-fluorouracil and hydroxyurea, respectively. No anomalies were observed in either cultured control (*n* = 106) or D-Phe-treated (*n* = 19) embryos.

BUdR/anti-BUdR

It was found that, within individual treated embryos, there are marked differences in the proportion of cells labelled with BUdR along the rostrocaudal axis in both the segmental plate and in the somites. Throughout the embryos, regions containing a high proportion of labelled cells are separated from each other with regions with a much lower proportion of labelled cells. This pattern is similar to that observed in the distribution of labelled cells after ^{3}H-TdR pulse-and-chase described above. Regions of neural tube adjacent to labelled somites also display a high proportion of labelled cells. Rapid fading of fluorescence prevented quantification of the proportion of cells labelled.

Immunohistochemical localization of heat-shock proteins

The results are shown in Fig. 7. We have used antisera against each of the three hsp classes (Schlesinger *et al.* 1982*b*) to stain histological sections of heat-shocked (*n* = 24) and untreated control (*n* = 15) embryos. Staining was uniform in every section of heat-shocked embryos: no differences in staining intensity were seen

between regions with somite anomalies and those without.

The time course of induction, as assessed by immunohistochemistry, differed for the three hsp classes.

(1) hsp 24

Unstressed control embryos exhibited barely detectable levels of hsp 24 expression. A substantial rise in the amount of labelling was seen after 1 h of post-heat-shock incubation at 38°C, while maximal levels of labelling were seen 3 h and 9 h after heat shock. However, by 24 h, hsp 24 expression had fallen.

(2) hsp 70

Unstressed control embryos displayed some staining with antiserum raised against this protein. A substantial increase above control levels of expression was observed only after 3 h following heat shock and this was still observed after 9 h. However, by 24 h of post-heat-shock incubation at 38°C, hsp 70 labelling had declined to control levels.

(3) hsp 89

Unstressed control embryos displayed some labelling with antiserum raised against hsp 89. An increase in the level of expression of this protein was seen immediately after heat shock, increased labelling still being visible 3 h and 9 h after heat shock. However, by 24 h, hsp 89 staining had decreased to control levels.

Characterization of heat-shock proteins

Each lane in the minigels used in these experiments
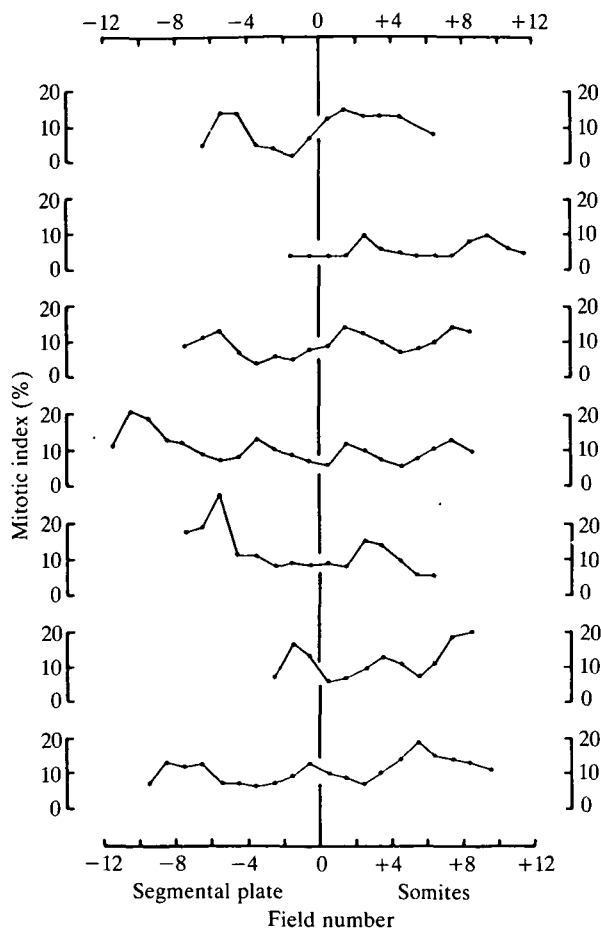
Fig. 4. Graphs of mitotic index (%, Y-axis) *versus* field position along the rostrocaudal axis (as defined in Fig. 3) of sagittally or coronally sectioned embryos. Each graph contains data from one representative section from one embryo. Position '0' on the X-axis is the rostral tip of the segmental plate.

Fig. 5. Histograms showing the frequency (Y-axis) and position (X-axis) of somite anomalies observed in 3-day chick embryos 24 h after treatment with either colchicine (COL), nocodazole (NOC), bromodeoxyuridine (BUdR), hydroxyurea (HU), 5-fluorouracil (5FU), or mitomycin C (MMC) at the concentrations described in the Methods. The last somite formed at the time of treatment is shown by an open arrow on the X-axis.

contained protein from the pooled segmental plates and somites of eight embryos from each experiment, and the protein concentration was the same in each lane; all experiments were carried out three times.
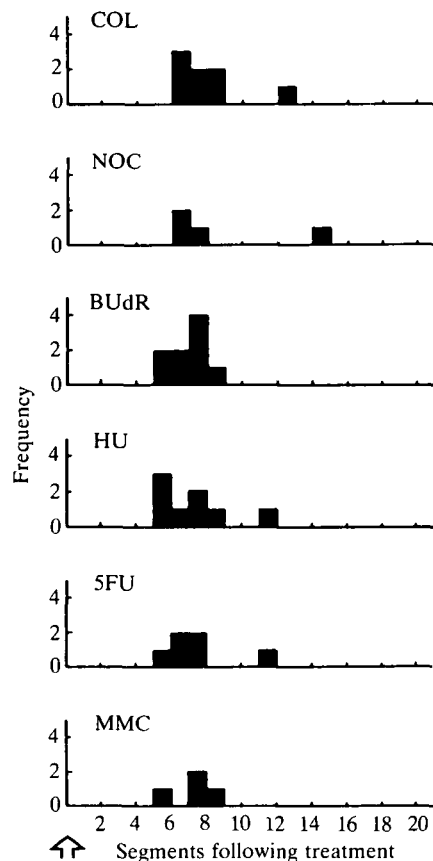
The temporal pattern of expression of the heat-shock response in 2- to 4-day chick embryos is shown in Fig. 8.

(1) *hsp 24*

Antiserum directed against hsp 24 recognized two bands on Western blots, one with an M_r of 22×10^3, the other an M_r of 24×10^3. Hsp 24 was not detected in non-heat-shocked 2-, 3-, or 4-day chick embryos, but rose immediately after heat shock. By 3 h of post-heat-shock incubation at 38°C, the level of expression was higher. However, by 48 h, it was barely detectable.

(2) *hsp 70*

This protein was expressed at low levels in unstressed 2-, 3-, and 4-day chick embryos. Hsp 70 expression increased immediately after heat shock, maximal levels being detected 3 h post-treatment. By 48 h, the level of expression was indistinguishable from that of controls.

(3) *hsp 89*

Expression was detectable at low levels in 2-, 3-, and 4-day chick embryos. Heat shock did not appear to induce its expression.

Fig. 9 shows expression of hsp resulting from treatment of 2-day chick embryos with either L- or D-Phe followed by 26 h of incubation at 38°C *in ovo*. Samples of L- and D-Phe-treated and control embryos show low level expression of hsp 70 and hsp 89 and no detectable expression of hsp 24. Fig. 9 also shows the expression of hsp seen after treatment of 2-day chick embryos with BUdR *in ovo* followed by 24 h of incubation at 38°C in New (1955) culture. Both BUdR-treated and cultured control embryos displayed strong expression of hsp 24 and hsp 70 but low level expression of hsp 89.

Discussion

Experiments on amphibian embryos have provided evidence that heat shock causes a disruption of segmentation visible several hours after treatment (see Elsdale
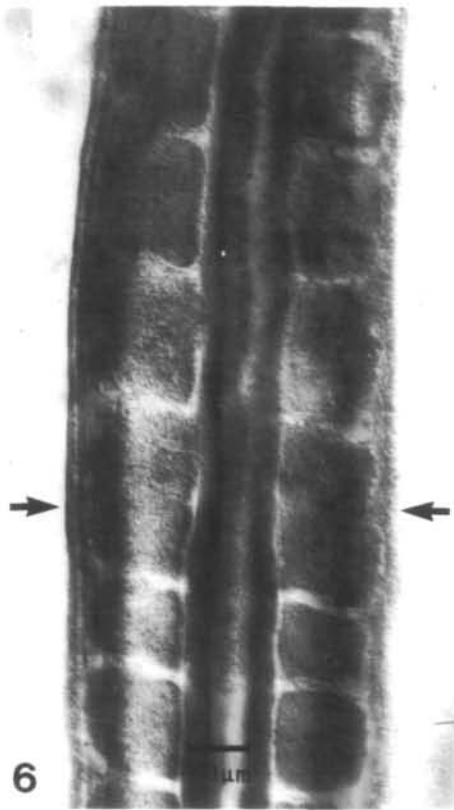
Fig. 6. Treatment-induced somite anomaly: embryo treated with 5-fluorouracil at the 15-somite stage; a bilateral fusion of somites 22–23 can be seen (arrows). Bar, 100 μm. Rostral to the top of the photograph.

et al. 1976; Cooke, 1978; Elsdale & Davidson, 1986; Veini & Bellairs, 1986). The time interval between the shock and its visible effect was held to reflect the time interval between the commitment of a particular group of cells to segment and the expression of their commitment (for review see Slack, 1983).

This interpretation must be re-evaluated in the light of more recent findings. In chick embryos, a single brief treatment with heat shock produces up to four discrete segmental anomalies: the first anomaly appears about 6–7 somites after that last formed at the time of treatment, and the remainder appear at about 6- to 7-somite intervals thereafter (Primmett *et al.* 1988). The observation that a *single* treatment can produce *multiple* segmental anomalies argues against the proposal that the time interval between heat shock and its effect represents the *'timing of segmental determination'* (see Slack, 1983), since, by definition, cells can only become committed once. Instead, we suggested that the periodicity of these anomalies reflects some underlying cyclic event (see Primmett *et al.* 1988), perhaps associated with the cell division cycle.

Since somites form at an approximate rate of about 1·5 h per segment (Menkes *et al.* 1961), the 6- to 7-somite interval corresponds to approximately 9–10 h. We would therefore expect that, if our suggestion is correct, the duration of the cell division cycle in cells destined to segment at the same time will be close to this value.

The cell division cycle in paraxial mesoderm and somites

The results of the ^{3}H-TdR pulse-and-chase experiment confirm these predictions: in 2- to 3-day chick embryos, the duration of the cell cycle in cells constituting the presomitic mesoderm, the newly formed epithelial somites and even the more mature mesodermal segments is 9·5 h.

### Methodology of [^{3}H]thymidine pulse-and-chase experiments

Our measurements of PLM *versus* chase time differ somewhat from the theoretically expected curve (see review by Steel, 1973). The PLM at 2 h after labelling is not zero, as might be expected. This may be due to experimental error, causing us to count some cells as being labelled mitoses when they were not, or it may reflect the possibility that a small proportion of the population (about 12 %) have a very short G_2 phase. Both possibilities are likely, and we expect that both contributed to our results.

It was often difficult to identify a cell as being in mitosis when it was heavily labelled and, on occasions, background grains may have led us erroneously to score some unlabelled dividing cells as being labelled. These considerations also explain why, at about 10–12 h chase time, our values do not approach zero. These factors, however, only affect the magnitude of the PLM and do not have an effect either on the position of the peaks or on our estimates of cell cycle transit time.

The duration of the G_2 phase of the cell cycle may be anomalous for two reasons: first, because its duration in the majority of cells (about 4–5 h) appears to be long as compared to other systems studied (e.g. Minkoff, 1984: at stage 24 in the facial mesenchyme of the chick embryo, the G_2 phase is about 3 h within a total cell cycle time of 12 h). Second, it may be that, as suggested above, a small proportion of the cells in the segmental plate and somites have a much shorter G_2 phase than the majority of the cells in these tissues. Despite these variations in the duration of G_2, however, the second peak seen in our curves of PLM *versus* chase time is fairly narrow, suggesting that those cells that became labelled at the same time maintain a certain degree of cell cycle synchrony.

Evidence of cell synchrony of somitogenic cells

Experiments on chick (Ozato, 1969; Stern, 1979) and mouse (Snow, 1977) embryos during gastrulation indicate that mesodermal cells of the primitive streak exhibit cell cycle synchrony. There is evidence suggesting that this sychrony might be maintained in later stages of development: Stern & Bellairs (1984) examined the segmental plate of early-somite-stage chick embryos and observed an elevated mitotic index at the caudal and rostral ends of the plate. From these findings, we would expect that those cells destined to segment together exhibit cell division synchrony.
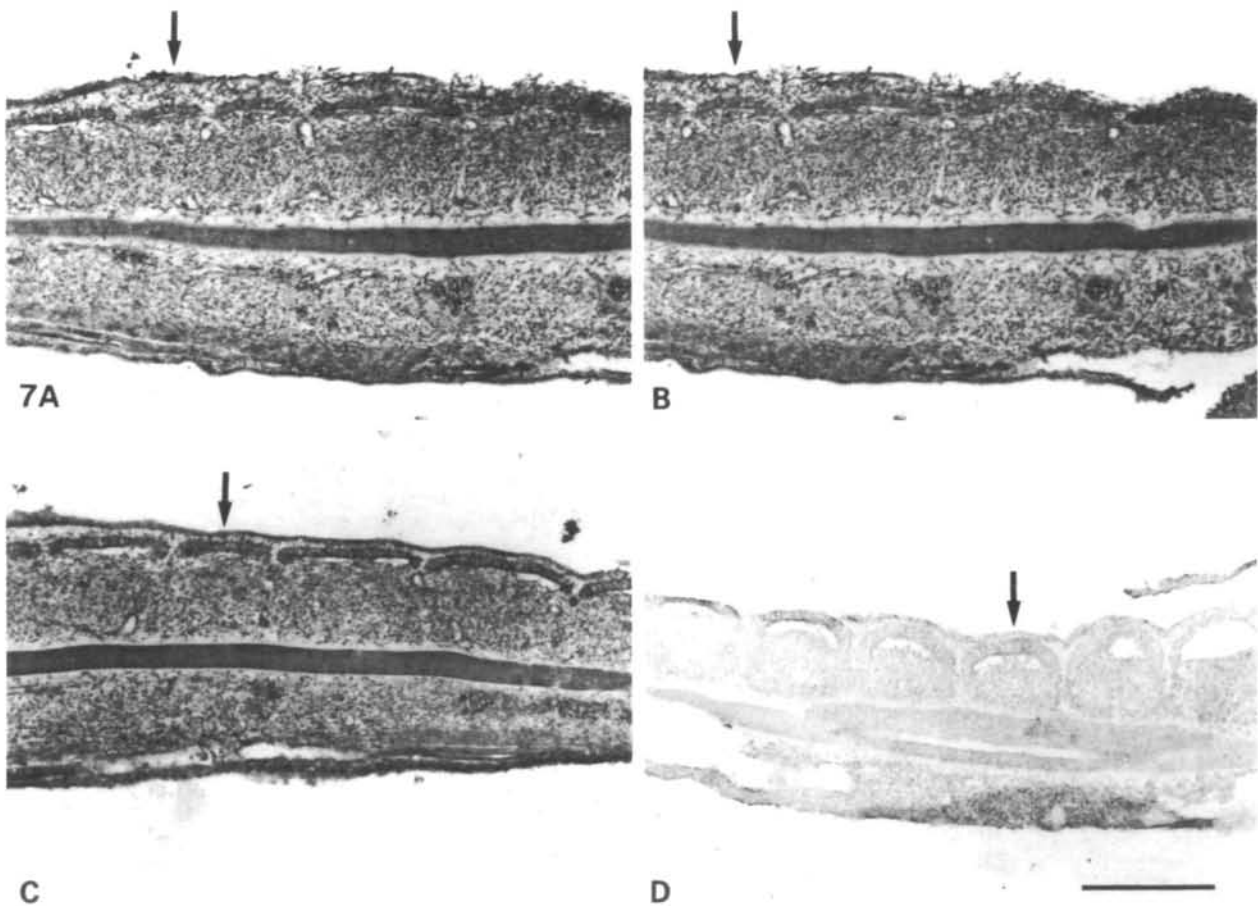
Fig. 7. Immunohistochemical localization of hsp in histological sections from chick embryos heat shocked on the 2nd day of development and fixed 24 h later. A treatment-induced abnormal somite (arrow) is visible in each of the sections. (A) Stained with antibody against hsp 24; (B) stained with anti-hsp 70; (C) stained with anti-hsp 89; (D) control (primary antibody omitted). Bar, 100 μm.

Along the rostrocaudal axis, we have observed discrete regions containing a high proportion of labelled cells, separated from each other by more elongated regions containing a markedly lower proportion of labelled cells. We have illustrated the spatial arrangement of such regions of high labelling index by plotting labelling index *versus* field number *versus* chase time (Fig. 3). The resulting three-dimensional graph reflects the passage of a labelled cell population through the different stages of somitogenesis. The interval between adjacent peaks on this graph equals 6–7 segments. The segmental plate of chick embryos contains about 12–14 presumptive somites (e.g. Packard & Jacobson, 1976), which is therefore equivalent to two entire cell cycles.

Since the interval between anomalies corresponds to one cell cycle, if the cell cycle itself is important in allocating cell populations destined to segment together, we would expect that interference with the synchrony of the cells will lead to segmental anomalies comparable to those observed after heat shock.

Cell cycle inhibitors cause segmental anomalies

We have used a variety of M-phase (colchicine and nocodazole) and S-phase (BUdR, hydroxyurea, 5-fluorouracil and mitomycin C) inhibitors to investigate whether they are able to mimic the anomalies produced by heat shock (Primmett *et al.* 1988). We have found this to be the case. All of these chemical agents produce anomalies comparable to those seen previously (see Figs 5, 6).

L-Phe treatment also generates similar anomalies. This is perhaps not surprising since heat shock and interference in L-Phe metabolism have both been shown to arrest cells in M phase (Mueller & Kajiwara, 1966; Miyamoto *et al.* 1973) and to cause somite anomalies in chick embryos (Palén & Thörneby, 1981; Primmett *et al.* 1988).

We can argue that the effects produced by all these treatments are not due to cytotoxicity; all the inhibitors were used at concentrations one to two orders of magnitude lower than those observed to cause cell death in 2- or 3-day chick embryos in culture (results not shown). At these low concentrations, the predominant effect of such agents is likely to be an inhibition of proliferation (see Scott, 1977).

It is also unlikely that the chemical agents affect
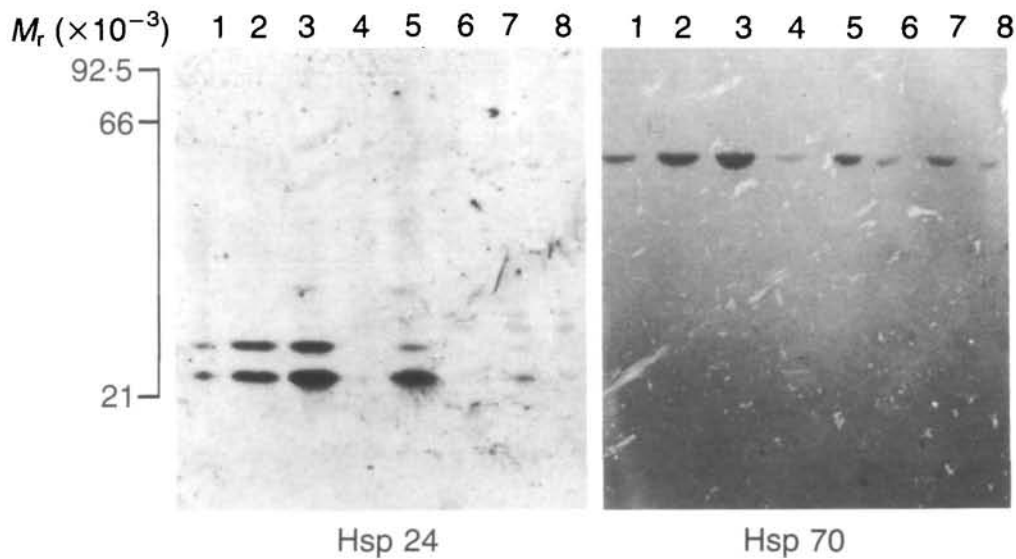
Fig. 8. Immunoblots showing the onset and decay of expression of heat-shock proteins hsp 24 and hsp 70 in heat-shocked chick embryos. Lane 1 = 0 h, lane 2 = 1 h, lane 3 = 3 h, lane 5 = 24 h, lane 7 = 48 h after heat shock. Lane 4 = 0 h, lane 6 = 24 h and lane 8 = 48 h non-heat-shocked controls. The scale on the left shows reference relative molecular masses ($\times 10^{-3}$), determined from radioactive standards run on the same gel.
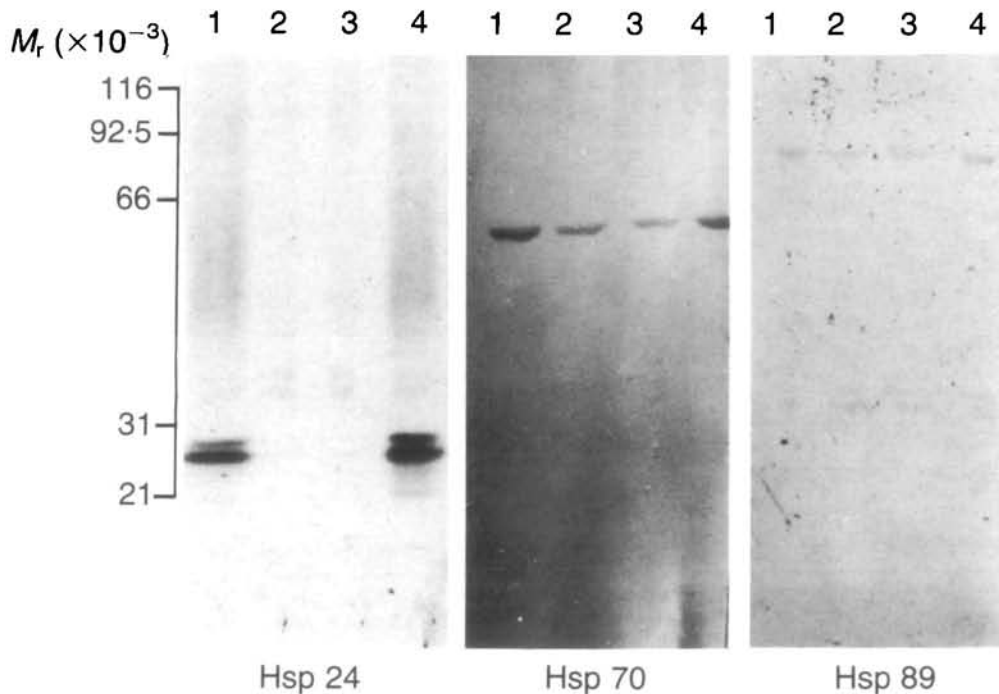
Fig. 9. Hsp 24, hsp 70 and hsp 89 expression resulting from treatment of 2-day chick embryos with either L- or D-Phe followed by 26 h of incubation at 38°C *in ovo* or BUdR followed by 24 h of incubation at 38°C in New (1955) culture. Lane 1 = cultured controls, lane 2 = L-Phe, lane 3 = D-Phe, lane 4 = BUdR (in culture). The scale on the left shows reference relative molecular masses ($\times 10^{-3}$), determined from radioactive standards run on the same gel.

somitogenesis through a specific effect on a cellular component that plays a critical role in somite formation, because they all act in very different ways. For example, colchicine and nocodazole inhibit microtubule polymerization (Wilson, 1977), BUdR inhibits thymidine incorporation into DNA (Stockwell, 1979), hydroxyurea inhibits ribonucleotide reductase activity

(Herken, 1980), mitomycin C causes DNA strands to cross-link (Murphy, 1960), and 5-fluorouracil inhibits pyrimidine synthesis (Wilson, 1977).

Another possibility must be considered: that heat shock and the chemical agents used all have a stressing effect on the embryo, and lead to the production of heat-shock proteins (hsp).

hsp expression does not correlate with segmental anomalies

According to Schlesinger *et al.* (1982*a*), the activation of heat-shock genes occurs as a universal response of cells to various kinds of perturbation. The induction of heat-shock proteins (hsps) may be dependent upon the binding of specific transcription factor(s) to the promoter regions of heat-shock sequence elements (e.g. Kelly & Schlesinger, 1978; Parker & Topol, 1984).

Chick hsp fall into three broad categories with apparent molecular masses of 24, 70, and 89×10^3 (Schlesinger *et al.* 1982*a*). Proteins with immunological and sequence homology to hsp have been designated heat-shock constitutive proteins and have been found to be expressed in a variety of non-heat-shocked animal tissues (Schlesinger *et al.* 1982*a*). The function of constitutive hsp expression is unknown.

In our experiments, we observed some expression of proteins that reacted with antisera against hsp 70 and hsp 89 in untreated. 2- to 4-day chick embryos, but almost undetectable constitutive expression of the $24 \times 10^3 M_r$ class of proteins. We find that heat-shock treatment of 2-day chick embryos strongly induces the expression of two classes of heat-shock proteins: hsp 24 and hsp 70. In our Western blots, hsp 24 was recognized by the antiserum as two bands with apparent molecular masses of 22 and 24×10^3. This is consistent with the finding that chick muscle cells have a $22 \times 10^3 M_r$ protein that cross-reacts with, but is not identical to, chick hsp 24 (Schlesinger *et al.* 1982*b*).

Are the periodic segmental anomalies observed in the chick embryo after treatment with heat shock, L-Phe, or mitotic inhibitors (e.g. BUdR) due to induced hsp expression in affected cells? If this is the case, one might expect that the pattern of such expression should match the pattern of these anomalies. However, we find that heat-shock-induced hsp expression does not exhibit a discrete or periodic distribution in histological sections of treated chick embryos; rather, heat shock appears to stimulate hsp synthesis in all cells throughout the embryo.

Still, it is possible that although all cells express hsp following heat shock, hsp production may be responsible for developmental abnormalities, for example by affecting cells that are in some critical phase of the cell cycle. In culture, BUdR-treated embryos exhibit anomalies and control embryos do not; however, both BUdR-treated and control embryos exhibit induced hsp 24 and hsp 70 expression. *In ovo*, excess L-Phe-treated embryos exhibit anomalies and embryos treated with D-Phe (i.e. the non-metabolic isomer) do not; however, neither L- nor D-Phe causes induction of hsp expression. Therefore, we can only conclude that hsp are not directly responsible for the pattern of segmental anomalies observed after heat shock or a variety of chemical treatments.

A simple model for the control of the timing of segmentation

Based on the findings reported in this paper, we can propose a simple model, summarized in Fig. 10, corre-
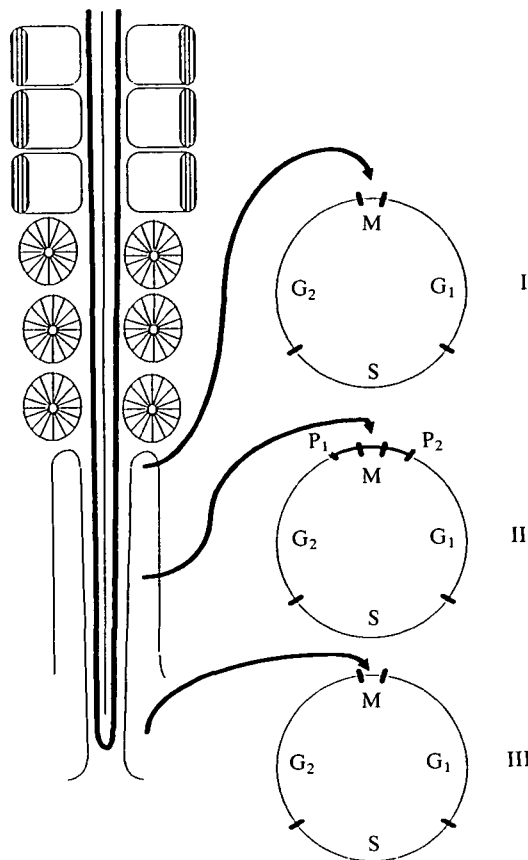
Fig. 10. Diagram of the model proposed, correlating stages of the process of somite formation with stages of the cell division cycle. Three cell cycles are shown, labelled I, II and III. The mitotic division of the first of these occurs at about the time of somite formation, while cells at cycle III will traverse two further full cycles before taking part in segmentation. Cells are proposed to become apportioned to a particular somite during cycle II, which precedes somite formation by one full cycle. This grouping of cells into presumptive somites is suggested to occur by virtue of the existence of two special points, P_1 and P_2, which might be situated on either side of the M phase of that cycle. The time interval between P_1 and P_2 is proposed to be 90 min, and to equal the time taken by one pair of somites to segment. 'Pioneer' cells arriving at P_2 would signal to other cells situated between P_1 and P_2, but not to those in other positions of the cell cycle; such a mechanism would group those cells that are situated within this time window.

lating the process of somite formation with the cell division cycle. The model makes the following assumptions:

(1) The time interval between the segmentation of consecutive segmental precursor populations (100 min) is equal to about 1/7 of the cell division cycle.

(2) There is some degree of cell cycle synchrony between cells destined to segment together.

(3) A short time before segmentation, those cells destined to segment together increase their adhesion to one another, regardless of their position within the segmental plate.

(4) This increase in cell adhesion always takes place

at the same time point of the same cell cycle, a fixed number of cell cycles after that cell population becomes committed as somitic. The segmenting cells can sort out from cells that are not yet competent to form a segment, that is, from those destined to form other segments.

To account for the rhythm of somite formation, a degree of hysteresis must be postulated. Hysteresis could be achieved, for example, if there were two special time points around the time of the last but one mitotic division before segmentation (cycle II in Fig. 10). The first of these (P_1) might occur towards the end of the G_2 phase of that cycle, and the second (P_2) would be close to the start of the next G_1 phase. The time interval between the two points should be 1·5 h (100 min). The synchrony between those cells destined to segment together is not perfect, however, and some cells will arrive at P_2 earlier than others. These 'pioneer' cells might produce a signal, to which any cell situated between P_1 and P_2 would respond by increasing its adhesion to its similarly responding neighbours. This mechanism punctuates the pattern, the size of each segment being determined by the number of cells that respond together at the critical time period.

In this model, the metameric pattern is generated by a process that does not rely on global signals, is linked to the cell division cycle, is independent of the position of the cells within the embryo and requires no separate oscillator with a period shorter than that of the cell division cycle. We have recently argued (Keynes & Stern, 1988) that amniote embryos are only capable of limited regulation of somite number. This model provides a simple mechanism for such regulation, since embryos smaller or larger than normal will contain proportionally smaller or larger numbers of cells distributed through the various phases of the cell cycle; therefore, correspondingly smaller or larger groups will be allocated to segment together. The model does not, however, account for the regionalization of individual somites leading to their differentiation into specific vertebrae, trunk dermal structures or skeletal muscles, although this may be possible with minor refinements. For example, groups of cells may count the number of cycles elapsed between some prior developmental event, such as their passage through the primitive streak and cycle II (Fig. 10), as a measure of their position along the rostrocaudal axis. It is interesting in this context that chick vertebrae appear to be regionalized in approximate multiples of 6–7: 14 cervical (excluding atlas and axis), 7 vertebrae with ribs (5 thoracic plus the first two lumbar), 5–6 sacral, 6 caudal and 6 coccigeal.

It is perhaps puzzling that inhibitors acting at different points of the cell division cycle (e.g. M-phase for colchicine and nocodazole, S-phase for mitomycin C, hydroxyurea, 5-fluorouracil and BUdR) cause the first of the anomalies to occur at about the same position relative to the somite that had just formed at the time of treatment. The model accounts for this paradox, since the gating is proposed to occur one full cell cycle before segmentation, and in that cell cycle only.

It may be significant to consider that the appearance of an intersomite boundary simultaneously defines the posterior half of the somite actually forming and the anterior half of the next somite to form. In other words, somite formation might be viewed as proceeding in *para*segmental rather than segmental steps (cf. *Drosophila*; Martínez Arias & Lawrence, 1985). We cannot conclude at present whether the '*segments*' defined in the model above correspond to somites or to parasegments.

Conclusions

We have measured the duration of the cell division cycle in segmental plate and somitic cells as 9·5 h. Our results are consistent with the notion that those cells that are destined to segment together have some cell cycle synchrony: we propose that the distance between consecutive anomalies caused by heat shock (6–7 somites; Primmett *et al.* 1988) corresponds to one cell cycle. Treatment with any of a variety of agents known to interfere with the cell cycle produces anomalies similar to those seen after heat shock. Although some of these treatments are shown to induce the production of hsp, our results indicate that hsp are not directly responsible for the pattern of anomalies observed. We therefore propose that transient arrest of the cell cycle by all treatments used is a sufficient explanation to account for the appearance and distribution of the segmental anomalies observed.

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