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An increase in cell–cell adhesion in the chick segmental plate results in a meristic pattern

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SUMMARY

Cell–cell adhesion in the anterior portion of the segmental plate plays an important role in the initiation of somitogenesis. Dissociated somites show a marked tendency to reassociate into somite-size clumps within a few hours after dissociation. Segmental plate cells show very little tendency to reassociate, even after 24 h in culture. The cells from the anterior portion of the segmental plate do show a tendency to reassociate. This supports the hypothesis that cell–cell adhesion between the cells in the anterior portion of the segmental plate plays a major role in the initiation of somitogenesis.

INTRODUCTION

It has been difficult to test the factors or mechanisms which have been proposed to regulate somite formation. The highly regular, meristic patterns of vertebrate somites have tempted many biologists to investigate this process (cf. Fraser, 1954; Spratt, 1955; Nicolet, 1971; Cooke & Zeeman, 1976; Elsdale, Pearson & Whitehead, 1976; Bellairs, Curtis & Sanders, 1978; Flint & Ede, 1982). Unfortunately many of the schema proposed could not be tested by critical experiments or gave contrary results to later experimentation. One hypothesis, however, can be tested by direct experimentation. This hypothesis includes cell adhesion as part of the mechanism of somitogenesis. Bellairs *et al.* (1978), using a Couette viscometer (Curtis, 1969) have obtained evidence that 'an increase in adhesiveness may play a role in somite segmentation. . .' (Bellairs *et al.* 1978). To understand fully whether an increase in cell adhesion causes somite formation, one must determine whether such a change occurs before or after the somite forms. The experiments reported here provide evidence that a change in cell–cell adhesion is initiated in the anterior end of the segmental plate before the somite forms. Thus, an increase in adhesion could play a causal role in somite formations.

The meristic pattern created through somite formation is morphologically simple; it is linear, symmetric and formed at regular and predictable intervals.

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The newly formed, or nascent, somites appear as seemingly homogeneous spheres of similar cells. Almost immediately there appears a stratified outer layer which forms a vesicle surrounding a loose clump of mesenchymal cells, the core cells (Williams, 1910). The outer layer is epithelioid, having been shown to possess junctional complexes and a basal lamina (Trelstad, Hay & Revel, 1967). Thus, within a very short time (a few hours in the chick embryo), an apparently homogeneous rod of tissue (the segmental plate) undergoes a dramatic and repetitive segmentation, followed by a rapid diversification and differentiation (Cheney & Lash, 1981; Lash & Cheney, 1982). The relative simplicity of the first event, i.e. segmentation, makes the process amenable to analysis and experimentation.

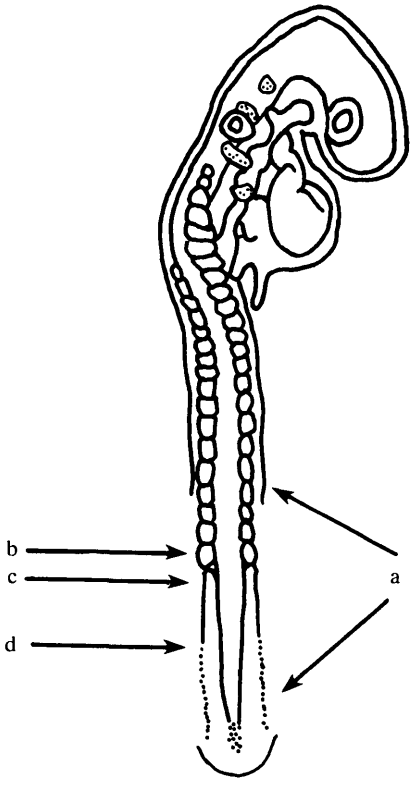
The segmental plate, as well as its subsequent somites, are suspended in a delicate meshwork of connective tissue (Ebendal, 1977; Belsky, Vasan & Lash, 1980; Chernoff & Lash, 1981). Medially, the plate is exposed to possible influences of the spinal cord and notochord, dorsolaterally is the epithelium, and ventrally the segmental plate impinges upon the extracellular matrices of the endoderm and dorsal aortae. The influence of these neighbouring tissues and structures is unclear. Some investigators find that these neighbouring tissues can influence somite formation (Spratt, 1955; Lanot, 1971; Nicolet, 1971), but others (Bellairs, 1963; Lipton & Jacobson, 1974; Packard & Jacobson, 1976) report that somites can form in isolated segmental plates. These latter observations, however, could be an expression of events triggered previously within the normal environment of the embry. Theoreticians have also approached the problem of somite formation, and a clock-oscillator wavefront model has been proposed (Cooke & Zeeman, 1976) to give a conceptual explanation for sequential segmentation. Such an explanation, however, says little of the events taking place at the cellular level.

The experiments reported here were designed to give information at the cellular level in what can be considered as a prime event in the initiation of somitogenesis, *viz*. cell–cell adhesion. A comparatively simple event such as the continuing acquisition of adhesivity between the cells in the anterior (cranial) part of the segmental plate can play a major role in the serial creation of a meristic pattern.

MATERIALS AND METHODS

Preparation of cell suspensions

Somites and segmental plates were removed from stage-15 (Hamburger & Hamilton, 1951) White Leghorn chick embryos. Some experiments were performed on HH stage-12 embryos to confirm the transition of a non-adhesive segmental plate cell (stage 12) to an adhesive somite cell (stage 15). All data in this report are from experiments on stage-15 embryos. The embryos were removed from the yolk and rinsed in sterile saline (Simms' balanced salt solution, SBSS; Simms & Sanders, 1942). With dissecting knives, the embryo was transected just



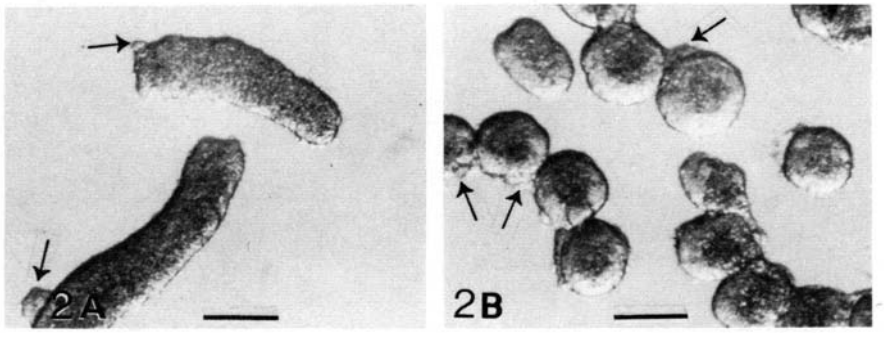


Fig. 1. Drawing of stage-15 embryo showing: (a) Region isolated for study, including the most posterior four pairs of somites and the segmental plate; (b) A newly formed, or nascent, somite; (c) The anterior region of the segmental plate; (d) The posterior region of the segmental plate.

Fig. 2. Isolated segmetal plate (A) and somites (B). Contaminating non-segmental plate or non-somite cells marked by arrows. Bar = 100 μm.

above the last four distinct somites (Fig. 1). After removal of the lateral mesoderm and extraembryonic membranes, the partial embryo trunk was pipetted into a 2·5 % trypsin solution (Grand Island Biological Co., Grand Island, New York) 1–3 secs, and quickly rinsed sequentially through three or four dishes of sterile SBSS. The somites and segmental plates were then dissected free (Fig. 2). Somites and segmental plates were collected separately in 35 mm Petri dishes containing sterile SBSS.

The tissues were dissociated in chymotrypsin (Worthington Biochemical Corp., Freehold, N.J.) (0·5 mg/ml in calcium–magnesium-free SBSS) on a rotary shaker (60 r.p.m.) in a humidified 5 % CO_2 incubator for 15 min. The tissues were transferred to small plastic centrifuge tubes for compaction and sequential rinsing in two to three changes of sterile SBSS. After rinsing, 2·0 ml of F12X cell culture medium (Daniel *et al.* 1973) was added to the tissues and they were triturated with a fire-polished Pasteur pipette that had been drawn to a diameter of 0·20–0·3 mm. The cells (somites and segmental plates) were distributed into 35 mm Falcon plastic Petri dishes containing 3·0 ml of culture medium. Cell concentrations ranged from 50–100 $\times 10^3$ cells/ml. Each experiment had three or four replicate dishes.

Assays for cell–cell adhesion

The cell suspensions were examined at 20× to 40× (dissecting microscope) and at 100×/200× (inverted phase microscope), and photographed at 'zero time,' 4–5 h, and 15–18 h. The 'zero time' observations were actually 20–30 min after the dissociated cells were immersed in the culture medium. Cell counts were made after 15–18 h, to determine the number of cells remaining unassociated.

In vitro *labelling of dissociated cells*

After the cells were dissociated, and just prior to placing them into the small Petri dishes, they were labelled with rhodamine isothiocyanate (RITC) (Pharmacia, Piscataway, N.J.), tetramethyl rhodamine isothiocyanate (TRITC) (BBL, Bethesda, Maryland), or fluorescein isothiocyanate (FITC) (Pharmacia, Piscataway, N.J.). RITC, TRITC, or FITC was added to the cells at concentrations of 5, 10 or 20 μg/ml in SBSS. The solution containing the cells was placed in a covered 12 × 75 mm plastic tube (No. 2054, Falcon, Oxnard, Calif.), and set on the rotary shaker as mentioned above, for 20 min. The cells were compacted with a brief (3 min) spin in a clinical centrifuge and the solution containing the fluorescent label was removed with a sterile Pasteur pipette. This procedure was repeated three times with sterile SBSS. After the final rinse, 2·0 ml of nutrient medium was added to the cells and they were counted. The labelled cells were mixed with unlabelled cells, as described below in Results. After 4 h and 18 h cells and/or aggregates were removed from the culture dish, compacted as during the rinsing procedure described above, and transferred to a glass slide

with a Pasteur pipette. The drop of medium containing the cells was ringed with Klearol white mineral oil (Witco Chemicals, New York), covered with a No. 1 coverslip, and examined for fluorescent cells using a Zeiss IV F1 epifluorescence condenser equipped with rhodamine and fluorescein filters. There was some transfer of rhodamine to the unlabelled cells. This was barely perceptive at 4 h, but more obvious at 18 h. All of the data in this report are from 4 h observations since at 18 h a weakly labelled cell should be either a labelled cell that had divided, or an unlabelled cell that had picked up a little bit of label. Limited observations were made on FITC-labelled cells because of toxicity even at the lowest concentrations. Cell counts of FITC-labelled showed a steady decrease in number during the 18–24 h observation period. RITC and TRITC-labelled cells showed no adverse effects during the 18–24 h observation period, and the cell count increased, indicating the cells were proliferating.

RESULTS

Reaggregation of isolated somite cells

In an effort to get a 'zero time' record of the cell suspensions, dissociated cells were photographed as soon as possible after being added to the culture dish. The interval of time was actually 20–30 min before the cells were photographed. Even at this early time, in 18 separate experiments, some of the somite cells had associated to form small clumps of two to five cells (Fig. 3). After 15 h of rotation, most of the cells had formed sizeable clumps (Fig. 4). When the medium was examined for the number of single cells remaining, there were too few cells to count on a haemocytometer. After 18–24 h of culture, the dish was full of somite-sized aggregates.

Reaggregation of isolated segmental plate cells

'Zero time' records of isolated segmental plate cells showed very few clumps of cells, and the rare clumps were usually 2–3 cells (Fig. 5). In 24 separate experiments, there was some variation in the number of cells in the aggregates, and sometimes the aggregates contained as many as 8–10 cells. The cause of the occasional large aggregate is unknown; it could be due to either contamination with other cells (e.g. lateral mesoderm) (cf. Fig. 2) or variations in different lots of foetal calf serum in the medium.

After 15 h of rotation, there was no perceptible change in the number or size of aggregates (Fig. 6). When this medium was analysed for the number of single cells remaining, the cell count was the same as the initial plating concentration at 4 h, but after 18–24 h there was a 20–50 % increase in the number of single cells. These results can be explained by the proliferation of non-aggregating cells.

In all instances where isolated segmental plate cells were tested for their ability to reaggregate, there was a consistent, but small number of aggregates formed.

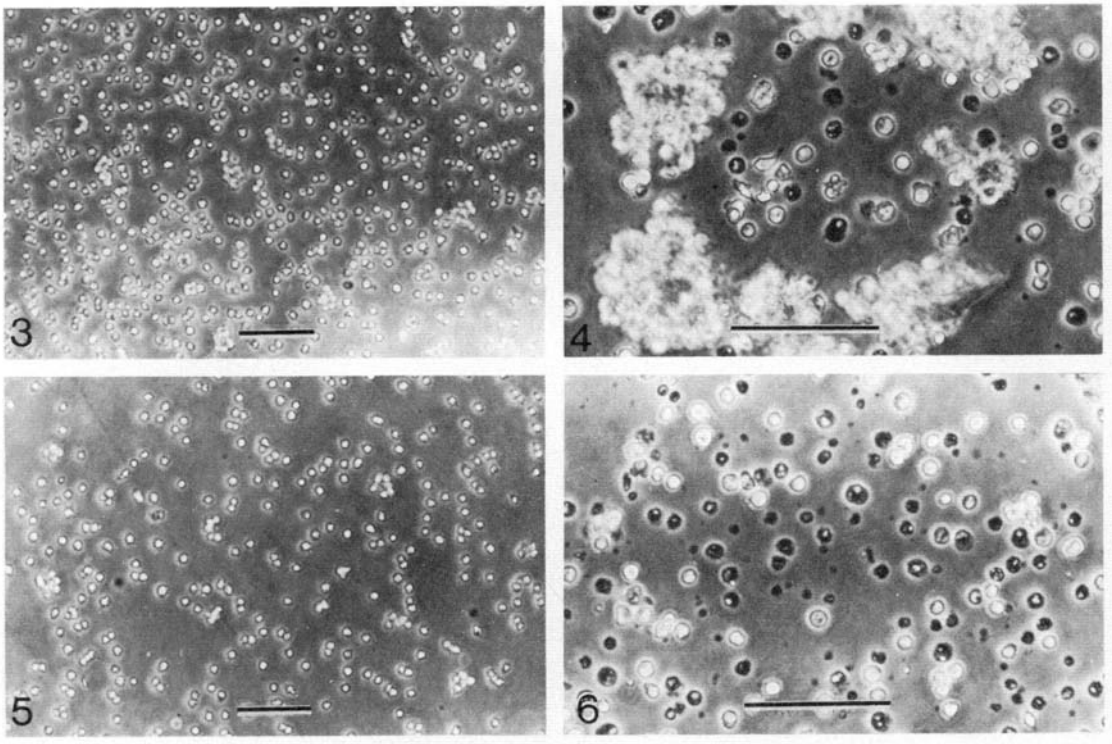


Fig. 3. Dissociated somite cells at 'zero time'. Although a few cells are touching other cells, it is predominantly a single cell suspension. Bar = 100 μm.

Fig. 4. Dissociated somite cells after 15 h on a rotary shaker. Most of the cells have aggregated into sizeable clumps. Bar = 100 μm.

Fig. 5. Dissociated segmental plate cells at 'zero time', predominantly a single cell suspension. Bar = 100 μm.

Fig. 6. Dissociated segmental plate cells after 15 h on a rotary shaker. There is little difference from the 'zero time' culture, with little evidence of cell aggregation. Bar = 100 μm.

The vast majority of the segmental plate cells showed no tendency to reaggregate after 18–24 h of culture.

In vitro *labelling of segmental plate cells*

If the cells in the anterior region of the segmental plate are the ones becoming adhesive during the initial stages of somitogenesis, then these cells may be the ones forming the few small aggregates when the entire segmental plate is dissociated and tested for the ability of cells to associate with one another. To test this possibility, the segmental plates were cut into two portions: an anterior portion (ant-SP) the length of approximately 1/2 somites (0·15–0·20 mm in length), and the remaining posterior portion (post-SP). After cell dissociation in separate dishes, the following labelling regime was followed: (a) labelled ant-SP

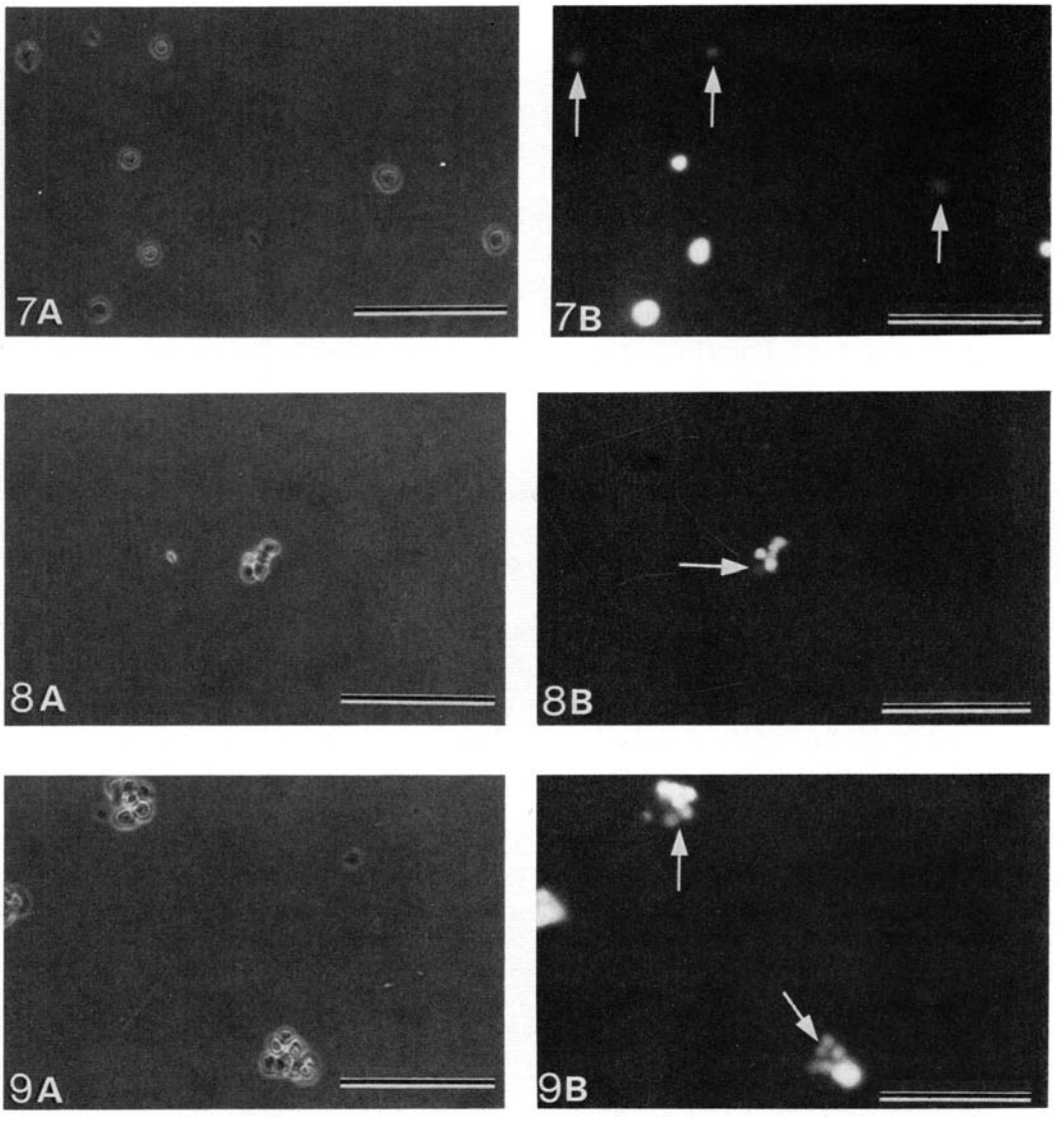


Fig. 7. A cell suspension of segmental plate cells. The anterior segmental plate cells were labelled with RITC. (A) Phase photomicrograph at 'zero time' (B) Fluorescent photomicrograph of same field, showing fluorescent RITC-labelled cells and unlabelled cells (arrows). Bar = 100 μm.

Fig. 8. Cells shown in Fig. 7 after 4 h on rotary shaker at 37 °C. (A) Phase photo of one small aggregate and a single cell. (B) Fluorescence photograph of same cells, showing that the single cell is unlabelled (i.e. from posterior segmental plate. The aggregate is composed primarily of RITC-labelled cells (i.e. from anterior segmental plate. One unlabelled cell is seen with the aggregate (arrow). Bar = 100 μm.

Fig. 9. Cells from a mixture of labelled (RITC) and unlabelled whole segmental plates after 4 h on rotary shaker at 37 °C. three aggregates and a single cell are seen in the phase photograph (A). B. Fluorescence photograph of same cell, showing that the aggregates are mixed, containing both RITC-labelled cells and unlabelled cells (arrows). Bar = 100 μm.

cells were mixed with unlabelled post-SP cells (repeated five times); (b) unlabelled ant-SP cells were mixed with labelled post-SP cells (repeated four times); (c) whole segmental plates were dissociated and labelled and mixed with unlabelled cells from whole segmental plates (repeated three times) (Fig. 7). All cell recombinations were examined after 4 h and the aggregates contained four to ten cells.

When labelled ant-SP cells were mixed with unlabelled post-SP cells, the aggregates were predominantly of labelled cells (Fig. 8). In a few instances an unlabelled cell was associated with an aggregate, but this was not a common occurrence. When unlabelled ant-SP cells were mixed with labelled post-SP cells, the aggregates were unlabelled and most of the single cells were labelled. There were a few instances where labelled cells were included in the unlabelled aggregates. Thus, in both of these experiments, the predominant cell type in the aggregates was derived from ant-SP cells. In an attempt to eliminate the labelling process as having an effect upon aggregation, whole segmental plates were dissociated and labelled, and mixed with dissociated unlabelled segmental plates. In these experiments, the aggregates should be predominantly of mixed (labelled–unlabelled) cell type. In Fig. 9 it can be seen that indeed the aggregates were of mixed cell type, presumably from the mixed labelled and unlabelled ant-SP cells. In all of the experiments listed above, labelling was done with RITC or TRITC at a concentration of 10 μg/ml. It was thought that an ideal experiment would be to mix RITC or TRITC cells with FITC labelled cells, and look for single-label or mixed-label aggregates. These experiments were performed, and the results were in agreement with the results listed above. FITC proved to be toxic to the cells, even at concentrations so low the fluorescent cells were barely distinguishable (5 μg/ml), and data from these experiments were not given. The results strongly suggest that cell aggregates formed from cell suspensions of segmental plates are predominantly of anterior segmental plate cells.

DISCUSSION

The acquisition of a meristic pattern during vertebrate somitogenesis can be partially explained by an increase in cell–cell adhesion in the anterior portion of the segmental plate. The segmental plate is a dynamic, constantly changing tissue where the anterior portion is sequentially partitioned as somites as new segmental plate tissue is added at the posterior end. Thus, during somite formation, the segmental plate is losing tissue mass in the anterior region and gaining tissue in the posterior region. The posterior portion is the 'youngest' region, and the anterior portion is the oldest region. It is during this transition from the posterior region to the anterior region that the cells become adhesive to one another. It is not known why the isolated segmental plate cells show so little reassociation after 18 h under the conditions we have used. It is possible that the concentration of fibronectin in the medium is too low to promote aggregation,

or that putative receptors on the cells' surface are absent. It is also possible that some sort of cell–cell or cell–extracellular matrix interaction is required for the expression of adhesion. These possibilities are under investigation.

Others have suggested that cell adhesion plays a role in somitogenesis. Until now, however, it was not known whether the demonstrated increase in cell adhesion was a cause or consequence of somite formation. The experiments reported here indicate that an increase in cell adhesion occurs in the anterior segmental plate and thus precedes somite formation. This observation adds considerable support to the hypothesis that cell adhesion plays a role in somitogenesis.

Somitogenesis is, however, a complicated, multistep process, and cell–cell adhesion appears to be an important factor only in the initiation of somite formation. It is important to distinguish between the initiation of somitogenesis and segmentation, which is the completion of somitogenesis. In this report we give additional information supporting the contention that adhesion plays an important role in the initiation of somitogenesis. A role for cell motile forces during somitogenesis has been proposed by Flint & Ede (1982). Supportive evidence has been reported by Chernoff & Lash (1981) on the role of cell movement in somitogenesis, and by Ostrovsky, Sanger & Lash (1983) on the role of actin during somitogenesis.

Nascent somites as well as older somites show a strong tendency for cell–cell adhesion. It is not known, however, whether the mechanism of adhesion is the same for somite cells as for segmental plate cells. The complicated cell movements during the development of the somite and subsequent diversification may require other methods of cell–cell associations. The experiments reported here support the contention that cell–adhesion in the anterior portion of the segmental plate is an important factor in the initiation of somite formation.

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