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The restrictive effect of early exposure to lithium upon body pattern in *Xenopus* development, studied by quantitative anatomy and immunofluorescence

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Summary

We have carried out an anatomical study of *Xenopus* larval and gastrula stages resulting from treatment of synchronous early blastulae for brief periods with Li^+. We confirm the proposal that such treatment causes a particular transformation, and partial elimination, of the normal body pattern. Coordinated restriction of pattern, without appreciable loss of cell number, is seen in all three germ layers. The distortion has been investigated by quantitative study of mesoderms at a standard stage, in relation to the normal fate map for mesoderm, and with the help of immunofluorescence on sections for somitic muscle and for blood. In the extreme syndrome, mesoderm arises from all around the blastula as usual, but is symmetrical and corresponds to that arising near the dorsal/anterior meridian of the normally specified egg or embryo with a large posterior subset of the normal pattern values thus missing. The effect is independent of any inhibition of archenteron formation or mesoderm migration (i.e. the cell mechanics of gastrulation) incurred by the treatment. It is also quite separate from a syndrome caused by more prolonged exposure to Li^+ during gastrulation. A small, but distinctive, anterior pattern region is also not expressed and, anomalously in relation to their general nature, these forms differentiate considerable blood tissue. We consider the implications of some details of the pattern restriction for our understanding of interaction in the normal development and propose that the Li^+ embryo is likely to be useful as a specific 'differential screen', in relation to the normal, during the search for those gene products that mediate initial regionalization of the body.

Key words: pattern formation, position values, mesoderm induction, convergent extension, gastrulation, blood formation, *Xenopus laevis*.

Introduction

Lithium has long been recognized to distort systematically the positional system that allocates the early material to the parts of the body, in a variety of embryos throughout the animal kingdom (Hörstadius, 1973). In some invertebrates, the effect has been relatively easy to understand, for each embryonic stage of exposure to Li^+ in the medium, as a disturbance of the proportions for the zones specified along a particular dimension of pattern. In vertebrates, the syndrome(s) caused have been harder to diagnose, perhaps because the anatomy is secondarily complicated by geometrical distortions in both the normal and the abnormal case, and also because of limitations on synchrony of the treated samples (e.g. Backstrom, 1954; Lehmann, 1945). A recent study has used synchronously fertilized groups of sibling *Xenopus* embryos (Kao *et al.* 1986), to reveal that a time window extends from the few-celled to the early-midblastula stage, during which time introduction of Li^+ indeed leads to systematic distortion of the normal allocation of material to axial structure. With suitable treatment, the distortion can be extreme, without appreciable cytopathology or underproduction of cell number (the system is dividing, but not growing, while pattern is established and then revealed in morphogenesis and differentiation). The body adopts approximately radial symmetry, with clear overallocation to those relatively 'dorsal' and

anterior levels of structure that are usually produced only far from the original meridian of sperm entry into the egg.

A subsequent study by Breckenridge *et al.* (1987) has corrected the false impression, which perhaps derives from the outer appearance of these embryos as well as from earlier literature, that Li^+ treatment is inhibitory to the formation of neural structures and/or to differentiation of tissue designated as neural. The paper was also concerned with the rate of Li^+ build-up in the compartments of the embryo and with effective intracellular concentrations. The study did not, however, appear to use precisely synchronous samples of treated embryos or to use conditions (dejellying of eggs) allowing the cleanest possible onset and offset of effective cellular exposure to the ion. Possibly for these reasons, the authors describe results in terms of disruption or disorganization, rather than any specific transformation, of the pattern of development within each germ layer of the post-gastrular embryo.

There are two motives for wanting a full morphological understanding of the lithium transformation in amphibian development. First, while the primary physiological action of the ion in distorting a positional signal is so far unknown, this is unlikely to remain so for very much longer. More sites of biochemical action of Li^+ are becoming known (e.g. Drummond, 1986; Berridge, 1984). There is thus potentially an important contribution to elucidating the physiology of the positional variable (Wolpert, 1971) which is set up across tissue in early development and subsequently guides the regionalization of the body. We would need to know whether Li^+ resets position value towards 'high', 'low' or specific intermediate levels. Second, even while the physiology of positional information remains obscure, a regime may be found that produces large, synchronous batches of a vertebrate embryo all proceeding towards formation of the same incomplete set of body territories. We would then have a 'differential screen' in relation to the normal embryo at each stage, with which to search for early, nonabundant gene products whose spatial patterns of synthesis mediate regionalization. Some such strategy is required if the understanding currently emerging from *Drosophila* early development is to be emulated for a group of organisms lacking its rich history of classical genetics.

The present paper offers a quantitative and qualitative anatomical description of *Xenopus* early-induced Li^+ syndrome that confirms, but also modifies, the diagnosis of Kao *et al.* (1986). It also confirms the time window in development, and optimal concentrations and periods of treatment that produce the fullest pattern restriction with the minimum cellular toxicity. The full syndrome, or limit form, involves production of massive, internal and synchronously developing notochord tissue but little or no adjoining somite in a near normal-sized mesoderm. The CNS pattern comprises radially arranged mid- or hindbrain but no forebrain or spinal cord. The progressively attenuated syndrome involves approach towards more normal anteroposterior elongation and gradation in differentiation schedule of the notochord. This is accompanied by development of spinal cord and of anterior partial subsets of the normal series of somite segments. Pronephros, heart and finally forebrain induction are restored to the pattern, the last not until almost normal development is reached. These findings are discussed in terms of the normal construction of the axis, of possible inductive and suppressive interactions between tissue types within mesoderm, and of nonequivalence (Lewis & Wolpert, 1971) or body-positional coding that is independent of tissue type. It should be mentioned, in relation to the entire results and discussion in this paper, that parallel studies in progress make it appear likely that Li^+ modulates the responses of tissue to inductive signals in making mesoderm, rather than affecting the array of such signals from vegetal regions as, for instance, does u.v. irradiation of the egg.

Materials and methods

Batches of synchronously fertilized *Xenopus laevis* embryos were obtained by standard procedures. Eggs were flooded after 10 min with 10% NAM (Slack, 1984; a balanced phosphate-buffered frog saline, henceforth NAM/10). A temperature of 20°C was used from then until the end of all lithium treatments. Between 2 and 3 h postfertilization (2- to 8-cell stage), eggs were dejellied by gentle swirling for 5 min, then passage through a wide-mouth pipette, in 2% cysteine HCl in distilled water brought to pH 7·9 with NaOH. They were briefly washed twice, then evenly dispersed for development in NAM/10.

Lithium treatment

Neither complete systematic optimization nor plotting of internal lithium concentration (see Breckenridge *et al.* 1987) was carried out. Overtreatment caused too high an incidence of arrest at gastrulation and death after cytolysis of the yolky endoderm, but did not alter the syndrome among survivors. Sensitivity for lethal and cytostatic effects seems to vary more between egg batches than does that for transformation of morphogenesis. Perhaps the ideal 'preparation' for production of the syndrome is to take synchronous embryos from, say, three females, and treat batches at the 64–128-cell stage with 0·125 M-LiCl in NAM/10 for 45 min, or 0·25 M-LiCl in NAM/5 for 9–12 min, followed by wash and further development for 45 min in a dish of NAM/3 (50 ml for 100 embryos) before return to NAM/10. Eggs from at least one female should then give >70%

incidence of high grades of the syndrome, with <20% embryos removed at the end of gastrulation with significant cellular damage.

Observation of embryos

In some experiments, entire batches of treated eggs and synchronous controls were inverted under NAM/10 with 5% Ficoll to prevent gravity-driven rotation, as late blastulae, for observation of externally visible gastrulation activity. All embryos were kept at normal room temperatures until standard control stages 32+, (29–30 somites segmented) or else 40 (Nieuwkoop & Faber, 1967). Examples typifying the appearances of the syndrome and controls were then fixed in 10% formalin + 2% acetic acid in 50:50 ethanol:NAM at room temperature for 48 h, then in 4% buffered formol saline for several days before dehydration and double wax embedding *via* celloidin/methyl benzoate and cedarwood oil. Sectioning was at 7 μm in the horizontal plane and staining was by Feulgen, with alcoholic light green and orange G counterstain.

Further examples were fixed for 2 h in the cold in 2% TCA, followed by 1 h in absolute alcohol and embedding *via* a wax:alcohol mix (two steps of 1 h) in PEDS low temperature (38°C) wax (Dale *et al.* 1985). These were sectioned at 10 μm and treated for indirect immunofluorescence with rabbit antisera to *Xenopus* muscle myosin and larval/adult globins.

Other embryos were explored with tungsten needles under the dissecting microscope, 5 min after the start of fixation in 2% potassium dichromate, 2% glacial acetic acid. This enables good separation and visualization of structures at stage 40, or of the disposition and thickness of cell layers in gastrulae. Quantification of the sizes of mesoderms and of the proportions of their cells in various structures was carried out on the Feulgen/light green/Orange G-stained section series, counting and assigning the mesodermal nuclei visible on every eighth section (Cooke, 1979*a*, 1981).

Results

Separate sensitive periods for pattern restriction and for production of head malformation

Fig. 1A,B are photographs and *camera-lucida* sketches of various degrees of the syndrome following exposure to LiCl during the early blastula sensitive period. During the pilot experiments, this series of body forms became familiar enough for an external classification of severity to be made and scored as grade 4, 3, 2, 1, with the normal body as 0 and the radially symmetric form as 4, along the lines of the classification of the syndrome that follows u.v. irradiation (Scharf & Gerhart, 1983). Even external inspection reveals that, where structure is restricted but recognizable, it is built on a correspondingly large scale within the material.

Six full time-course experiments were performed with closely similar results. Table 1 presents data from two such experiments where batches of around 30 embryos from the synchronous population were treated at intervals beginning approximately 1 h apart at 20°C. Three embryos were fixed near the midpoint of each treatment (i.e. 20 min after its start in experiment 1 and 5 min after the start in experiment 2) to record the progression of morphological stage. Treatment ending by the 8/16 cell cleavage rarely caused the syndrome. Treatment beginning after mid stage 7 for the long, low concentration version, or early stage 8 (512 cell) for the shorter high concentration version, rarely caused more than the attenuated syndrome involving a truncated but extensive series of somites and a slightly large head and deep 'chest' region (grade 1). The mean score of affected individuals, and the overall incidence, declines systematically from about the 128-cell stage to the onset of stage 8. The set of posterior axial structures produced then 'builds out' towards the normal pattern, starting from hind-brain or ear-vesicle level. Attenuated forms of the pattern restriction, apparently identical to those incurred by lateness of Li^+ exposure, are also produced by inadequate concentration and/or length of treatment during peak sensitivity.

It is not worthwhile to treat larger batches at shorter time intervals in order to produce pure populations of various intermediate forms. Nor indeed can any large, pure population of pattern-restricted bodies be produced by treatment and then sorting away of dying individuals as gastrulae. Lethality is apt to rise unacceptably, above say 30% of embryos treated, as the incidence of the pure syndrome rises above about 85% among survivors. But inspection of the form of gastrulae at control stage 10+ (see below) allows rapid sorting of a pure population of radialized forms with high efficiency. The circular, raised and band-like blastopore lip that goes with the fully restricted pattern (Fig. 1C) develops abnormally high in the marginal zone material, being near the equator and within the surface-pigmented part of the animal cap. Table 1 illustrates the important finding that the final body pattern restriction after early blastular treatment is quite independent of mechanical effects upon the schedule of blastopore closure. The exact timing of the synchronous, radially symmetrical formation of the initial lip is constant and characteristic of the pattern restriction (see Discussion), but subsequent blastopore closure can take two courses. There is a certain incidence of delayed and incomplete closure, but the alternative sequence of events is an extra rapid, vigorous and symmetrical progression to morphological stage 12, the closed blastopore. Both mechanical versions of gastrulation can accompany grade-4 or -3 pattern restriction. Any delay in closure registers only as a

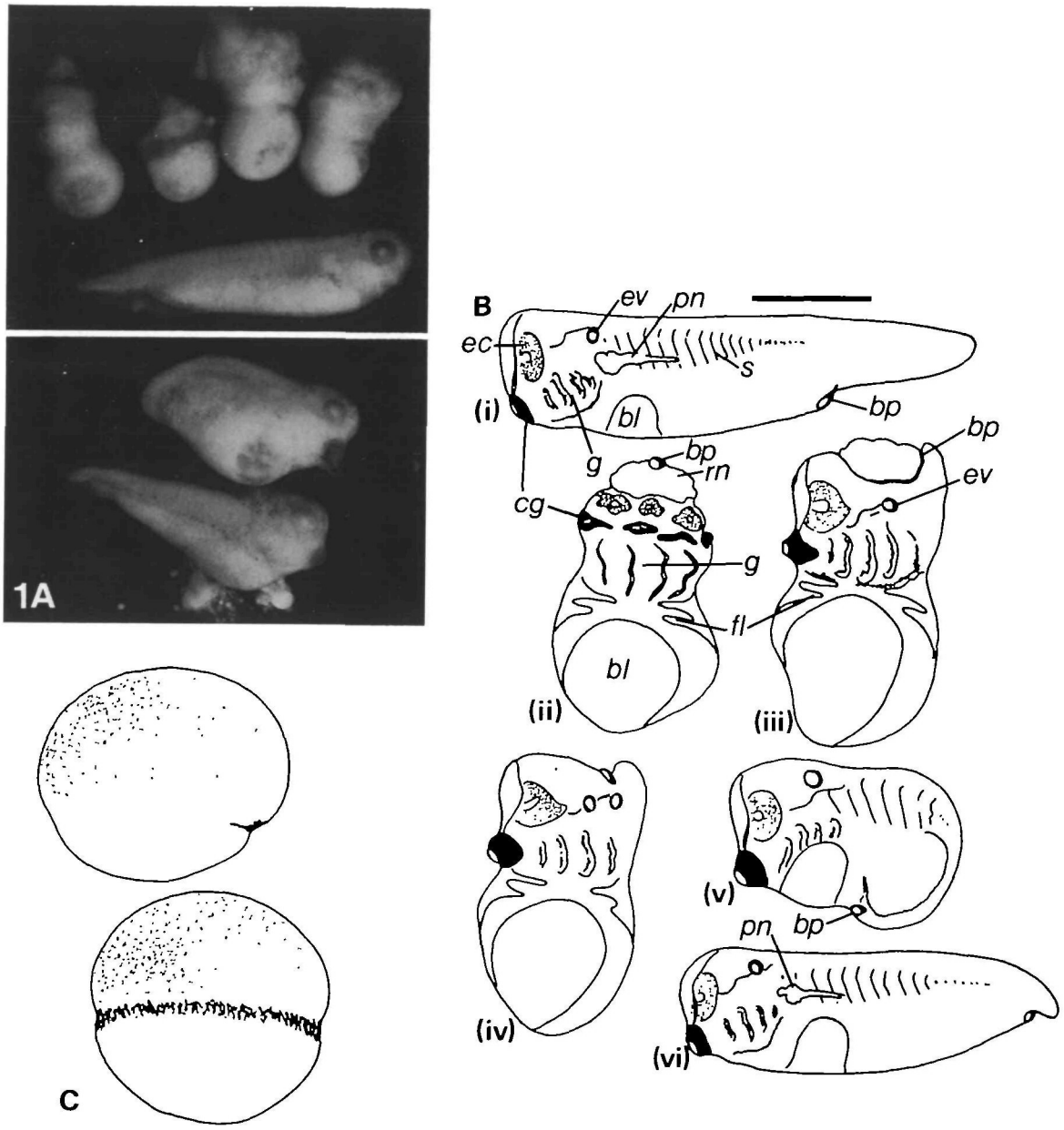


Fig. 1. Graded external morphologies following early blastular lithium treatment. (A) A group of Li^+-treated embryos at late tailbud stages, surrounding a control of normal morphology. The upper group of four have severe versions of pattern restriction (grades 4, 3) while the lower pair represent grades 2 and then 1, the attenuated versions. (B) Sketches of the external forms seen at stage 32. Reading from left to right and top to bottom, these are the normal; grade 4; the versions of grade 3 with incomplete (iii) and complete (iv) blastopore closure (see text); and a grade 2 (v) and grade 1 (vi) pair as in A. (C) Lateral views of a normal early gastrula stage 10 (upper) and of a synchronous embryo due to develop into a severe (grade 4) pattern-restricted form. Note the high, near-equatorial position and synchronous, ring-shaped development of the zone of blastoporal activity. Scale bar for the drawings, approx. 1 mm. *cg*, cement gland; *ec*, eyecup; *rn*, radial neuralized area; *ev*, ear vesicle; *g*, gill architecture; *fl*, prominent flange-shaped zone of gut morphology (see text); *bl*, original blastocoel cavity; *pn*, pronephros; *s*, somites; *bp*, blastopore or open blastoporal rim.

visible yolk plug instead of a nipple-like pore on top of the final form (Fig. 1B, two versions, grade 3).

There is an appreciable gap in sensitivity from stage 8 to 9+, followed by a new phase of sensitivity during gastrulation for production of a clearly different type of pattern defect. For this, a longer exposure to each Li^+ concentration is required, and the incidence is lower and more variable. Externally, synophthalmia or monophthalmia and/or an amorphous appearance to brain and gill region is seen, but with a normally elongated axis posteriorly. Histology reveals a solid mass of tissue with pyknotic nuclei in anterior brain

Table 1. *Exposure of synchronous siblings to LiCl at pregastrular stages*

Stage at midpoint of treatment	Sample size at control stage 12		Scores at control stage 32 in subsamples; see previous column	
	(1) Open yolk plug	(2) Normal or rapid yolk-plug closure	(1)	(2)
(a) *Experiment 1 0·12 M-LiCl for 40 min*				
4- to 8-cell	10	14	8 spina bifida full axis 2 normal	13 normal 1 dead
32-cell	18	13	16 grade 4, 1 grade 2 1 normal	9 grade 4 4 grade 3
64- to 128-cell	17	11	11 grade 4, 6 dead	7 grade 4 4 grade 3
256-cell (stage 7)	14	12	8 grade 3, 6 dead	5 grade 3 7 grade 2
512-cell	7	20	7 grade 3	4 grade 3 12 grade 2 4 grade 1
mid-stage 8	3	27	1 grade 2 2 dead	1 grade 2 1 grade 1, 25 normal
stage 9	5	25	4 synopthalmia +small head, 1 dead	23 normal 2 dead
(b) *Experiment 2 0·25 M-LiCl for 8 min*				
8- to 16-cell	3	17	3 spina bifida, but full or grade 1 axis	2 grade 1 15 normal
32- to 64-cell	6	20	4 grade 3 2 dead	1 grade 4, 10 grade 3 & 9 normal
128-cell	7	12	5 grade 4 2 dead	11 grade 4 1 grade 3
256- to 512-cell	14	13	7 grade 3, 2 grade 2 5 dead	9 grade 3 3 grade 2, 1 dead
mid-stage 8	11	20	9 grade 2, 1 spina bifida, 2 dead	2 grade 2, 9 grade 1, 9 normal
stage 9	7	23	5 synopthalmia +small head, 2 dead	22 normal 1 dead

Grading of Li^+ pattern restriction; 4, apparently radial symmetrical; 3, 'directional' head and brain structure but no apparent axis; 2, truncated body form, normal for first 5–10 somite segments; 1, truncated form with 10–20 somites.

and mesoderm, sometimes with notochord loss and truncated brain pattern. Treatment during gastrulation appears reliably to arrest morphogenetic movements, incurring delays of many hours in their completion that may do much to explain the anterior disruptions. This phenomenon is mentioned here only to distinguish it from the pattern transformation following early treatment that is the main subject of the study.

Quantitative anatomy by tissue type

In some egg batches, the two rounds of cleavage that follow blastular treatments at above 0·15 M-LiCl are noticeably delayed, yet by onset of gastrulation the cumulative cell cycle delay is insufficient to cause any detectably large-celled appearance in the pigmented animal cap. The full (grade 4) larval syndrome can develop without appreciable cell shedding or large-celled appearance. A small population of free, yolky cytolysing cells in the archenteron is, however, frequently seen.

Larval mesoderm can first be characterized by tissue types and proportions without reference to their arrangement and architecture. To do this already reveals certain striking features, notably the large allocation to notochord but small amount of somite tissue. Table 2 shows the relative overall sizes (sampling density = approx. 20% of the total) of mesoderm cell populations is one set of fully radialized pattern restrictions (grade 4) and one set of grade 3 restriction (heads with directionality only), together with the percentages allocated to notochord, somite muscle and other mesoderm, which in experimentals is overwhelmingly of branchial or lateral plate character (no pronephros or heart).

The mesoderm of the limit form (grade 4) is measurably reduced in cell population, but not nearly to the extent that would be the case if its highly restricted pattern (notochord and posterior head

Table 2. *Quantitative mesodermal anatomy of Li^+ embryos by tissue type*

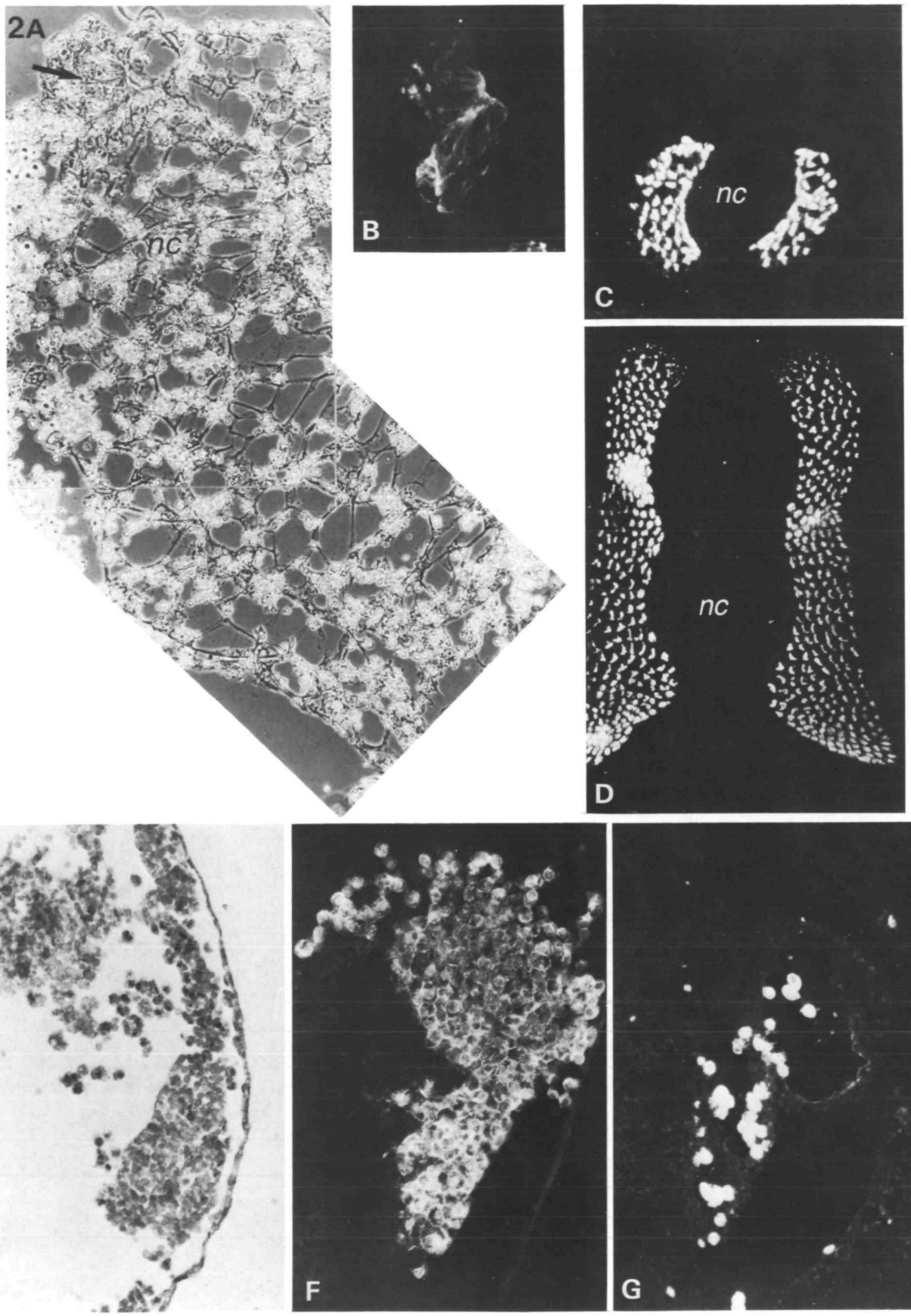
		Total of nuclei encountered on scanning every 8th section	Percentage allocated to		Remainder (uncharacterizable by tissue type in experimentals)
			Notochord	Somite muscle	
Set 1. Control	1	4522	4·6	34·9	60·5
	2	3980	5·1	37·6	57·3
	3	4609	4·3	33·1	62·6
Grade 4	1	2370	24·7	0·9	74·4
(limit form)	2	2758	27·6	1·2	71·2
	3	2974	23·9	1·0	75·1
	4	2073	28·4	0·8	70·8
Set 2. Control	1	5115	3·4	32·6	64·0
	2	4376	4·9	36·3	58·8
Grade 3	1	3112	29·0	6·2	64·8
(4 clear somites)	2	3401	29·3	5·4	65·3
	3	3796	27·1	12·2	60·7

The plane of section used for estimates of relative cell number was the horizontal for normal development and its equivalent in the Li^+ embryos. In seeing every 8th section in such material, some 20% of the cell nuclei are encountered.

mesenchymes only) was built on the normal scale. Cell number allocated to notochord can be four times the normal and yet be accompanied by only small wisps of somite tissue in the adjoining mesoderm. The immunofluorescence for muscle in Fig. 2A–D shows how the few somite cells present are related to the notochord in a way that exaggerates that normally obtaining in the ear vesicle region, where somites are very few-celled. The massive lateral plate is in keeping with this, since it is at such levels of the normal axis that lateral plate cell numbers are relatively the greatest. In larval forms of grades 3 and 2, total mesoderm is within normal limits despite the still greatly restricted pattern. Somite percentage builds up, though still below normal, as the form of the notochord elongates and increasing axial structure builds out (described in next section).

Dissection of late gastrulae and neurulae developing the radialized syndrome reveals that there is no true blastocoel remnant, free of mesoderm, as in normal development (see Fig. 5 and Discussion). Instead, the cavity underlying the cone-shaped endoderm mass, which finally hangs downward in gravity at the opposite end from the blastopore, comes to be occupied by mesoderm that has the appearance of blood islands under the light microscope. This releases many cells (Fig. 2E) whose nuclear morphology resembles immature blood before its release into the vascular endothelial spaces that form in normal larvae. The haemopoietic nature of this tissue was confirmed by immunofluorescence to *Xenopus* blood in control and in fully pattern-restricted material at stage 40 (Fig. 2F,G). It is, indeed, of considerable extent in the experimental embryo, but more resembles the *in situ* developing blood normal to early 30s stages than that circulating in normal stage 40, being of relatively dull fluorescence and compact, palisade structure. We can assume that substantial blood is formed in the Li^+ embryo, but that due to absence of certain other pattern parts, a continuous and circulating endothelial system is never found in which it can complete its maturation. Its presence is surprising in relation to the other effects on mesodermal pattern and we return to it in the discussion.

Fig. 2. Amounts of somite and notochord, and occurrence of blood, in fully pattern-restricted bodies. (A,B) Phase-contrast and immunofluorescent images for *Xenopus* somite muscle in a stage-40 grade-4 Li^+ body. Note massiveness of notochord (*nc*) and very small allocation to somite, whose position is indicated by an arrow on the phase picture. (C,D) Immunofluorescence for muscle in transverse sections of stage-40 normal development, at ear vesicle/hindbrain and trunk axial levels respectively. Note the small allocation of somite, in relation to the more constant-sized notochord allocation, at the former more anterior level. (E) Part of a 'horizontal' section (stage 40) through that portion of the fully pattern-restricted body that hangs downmost in gravity, showing cells resembling the normal immature blood of the mid 30s stage larva in Feulgen, light green and orange G staining. Note intense nuclear staining and free, nonadhesive appearance of the cells concerned. (F,G) Immunofluorescence for *Xenopus* larval and adult globins at control stage 40, in part of the grade-4 Li^+ body near that seen in E and in a grazing section near the heart of the normal larva, respectively. Note relatively massive development but immaturity of the blood tissue in F as opposed to the brightly fluorescing, circulating blood cells of G. Magnifications, (A,B,F,G) ×160, (C,D) ×100, (E) ×250.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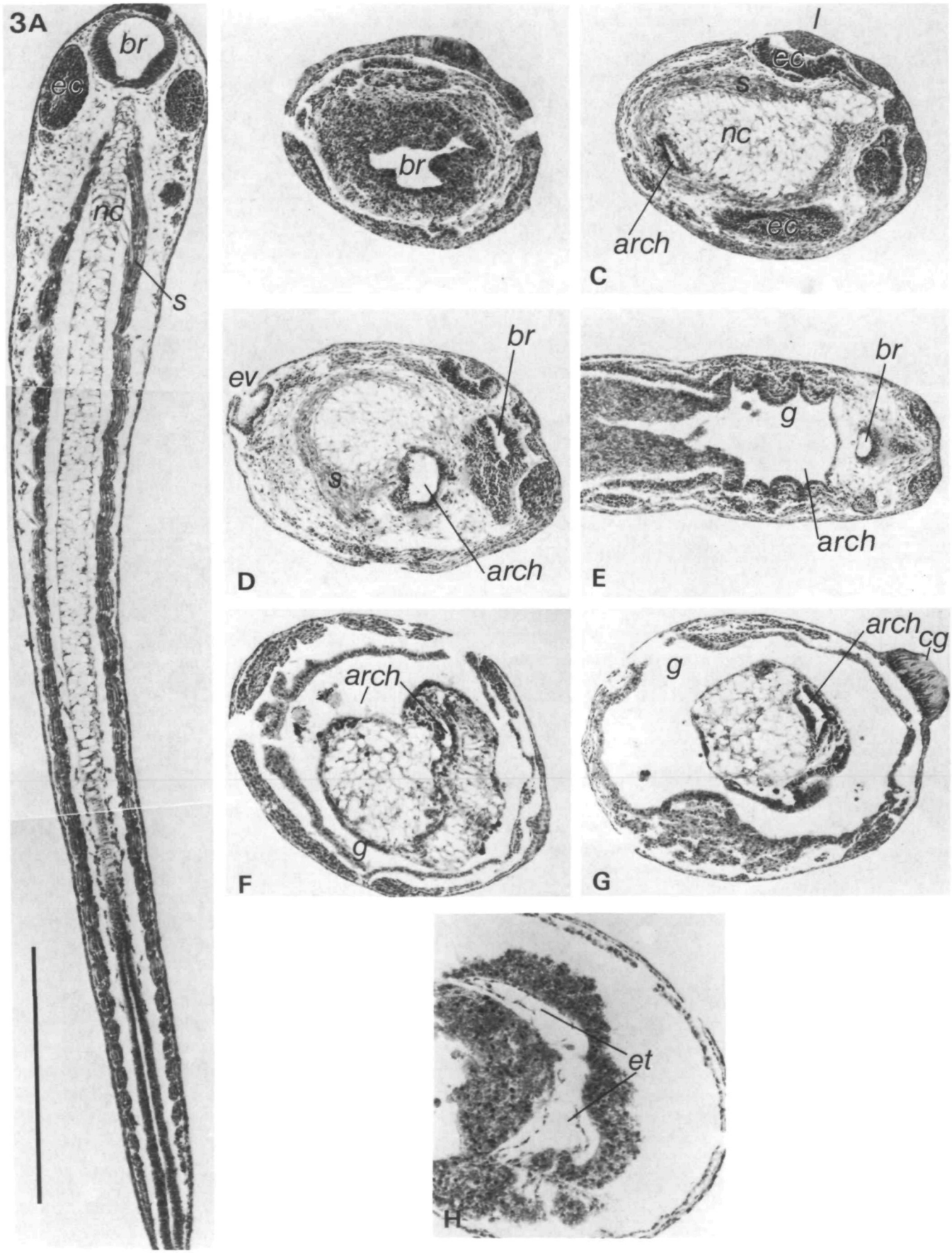
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The morphology of the full and the attenuated pattern restrictions

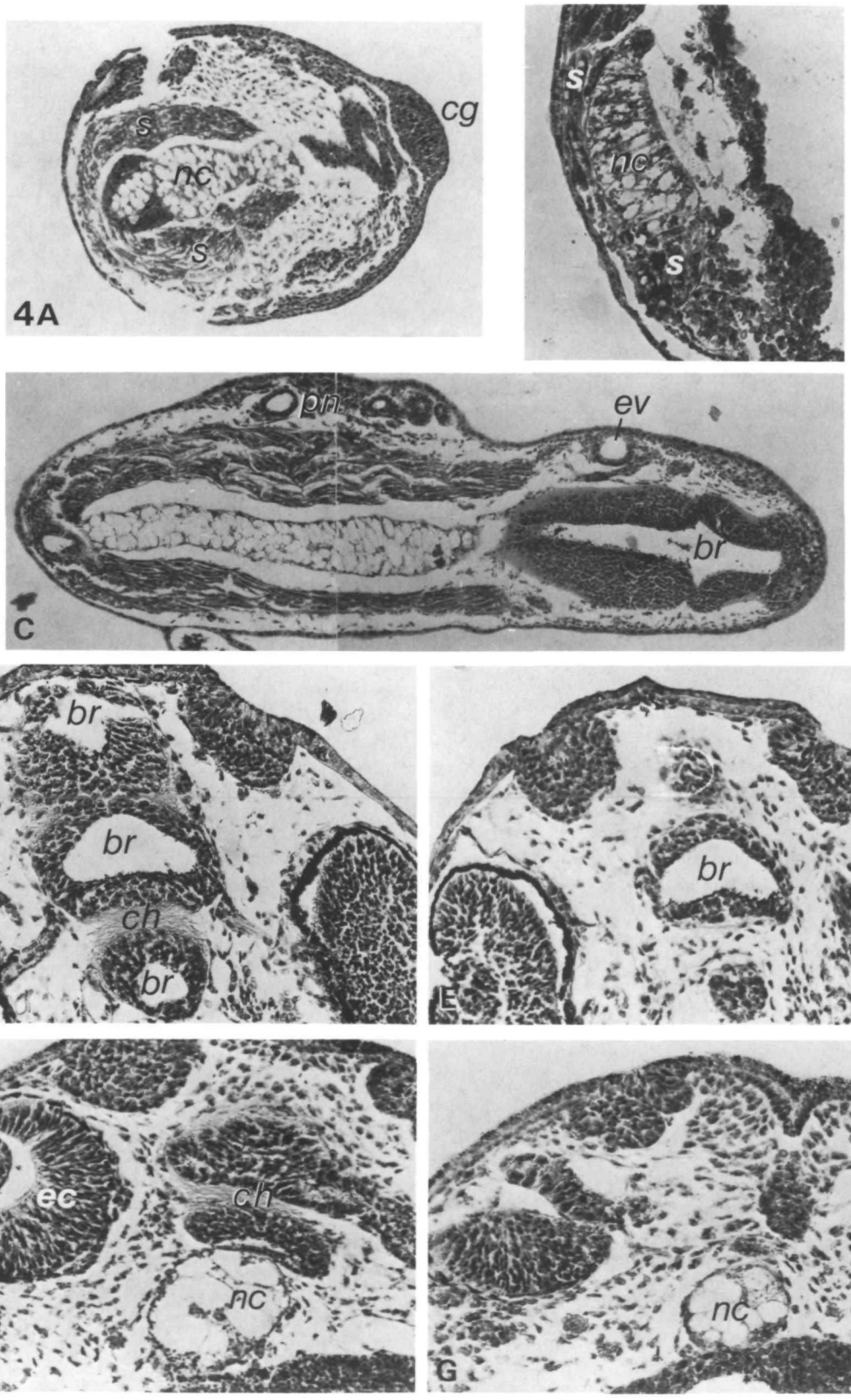
The photomicrographs of Fig. 3 show sections at various levels through the normal stage-32 body, the radially symmetrical, limiting version of the Li^+ syndrome and a directional but strongly pattern-restricted version (score 3). Those of Fig. 4 show significantly axial but truncated bodies that are typically seen after treatment near the midblastula stage (scores 2 and 1). Fig. 4D–G show sections through the optic chiasm level at the anterior notochord tip, then at 40–50 μm 'ventral' (i.e. in pattern terms, anterior) to this, in a normal and in a score-1 pattern-restricted body at stage 40. The latter photographs illustrate how the syndrome involves complete absence of the forebrain, a small but distinct domain in lower vertebrate development whose induction is normally caused by underlying prechordal mesoderm during gastrulation/neurulation, but which is never found in the Li^+ pattern-restricted body.

A plane of section equivalent to the horizontal in normal development is chosen to illustrate the syndrome, since it is a restriction more of anteroposterior than of dorsoventral pattern sequence. But in the limit case without axial pattern, the upper part of this section series is transverse with a central blastopore and crevice-like archenteron, which dives down through the neuralized area. The radially symmetrical pharynx is horizontal, and then the rest of the gut is laid out beneath this. Fig. 5B,C, derived from fix-dissection of normal stage-14 neurulae and their lithium-treated siblings, together with Fig. 5C,D, may help readers who wish to understand from a mechanical point of view the abnormal geometry that the embryo structure is led to adopt. We believe, however, that the final contents of Li^+ bodies are most meaningfully explained in terms of the production of a coherent but restricted subset of the normal body regions (Fig. 5A and Discussion). We briefly list the structures encountered in the limit form, then describe what happens as the restriction on pattern widens, the blastopore moves to a position describable as posterior to the neuraxis, and the mesodermal pattern 'builds out' to bring the gut structure into its normal relations with other layers. It is re-emphasized that a rather normal-sized total mesoderm is used to construct whatever is made, so that the more restricted the pattern, the larger the scale of construction seen.

The blastopore is surrounded by a turret-shaped mass of neural tissue whose pattern is scarcely diagnosable, except for a band of pigmented retinal tissue. In less fully radial cases, mid- and hindbrain regions become suggested by the subdivisions of the retina into cup-like masses that communicate with a central thick-walled cavity, and by clear ear vesicles, often more than two, but adjacent to a 'posterior' subdivision of the neural mass. Underlying these structures is a very large notochord territory of spherical or vertical peg shape, whose lower part may be wrapped with thin endoderm (archenteron roof?) but project into the wider archenteron cavity below. The latter cavity forms branchial bars and slits in radialized or anteroposterior bilateral array, which are invested with a heavy population of mesenchyme identical in appearance with that normal to the branchial region (see Fig. 3). The latter is believed to include neural crest and true mesodermal contributions. This mesenchymal region abutts directly on those boundaries of the notochord mass that are not wrapped in endoderm. A slit-like, separate minor

Fig. 3. Sections through severely pattern-restricted lithium bodies. (A) Horizontal section at notochord level through the normal stage-32 body; *br*, brain. (B) Section through the turret-shaped neural area and grazing the ring of cement gland formations, in a grade-4 Li^+ embryo. The evaginations from a central neural cavity and the peripheral formations with the appearance of eyecup indicate the diencephalic level of development. (C) A subsequent level of section and from a body showing somewhat more anteroposterior directionality. Note the massive notochord allocation, the more clearly retinal structures (more than two are typically seen), the narrow strips of unorganized somite material and the transversely sectioned tunnel of archenteron traversing the posterior 'side' of the notochord. The notochord shows no gradation of differentiation stage; *e*, eye; *l*, lens. (D) Similar section level to C but showing an ear vesicle in addition to eyecup and midbrain profiles, and a wider archenteron tunnel which is not passing 'posterior' to the notochord. (E) Horizontal section through the pharynx or gill architecture of the normal body. Note shape of the thin endodermal wall and character of the relatively massive investing mesenchymes. (F,G) Sections through the pharynx or gill region in two almost radial pattern-restricted bodies. Note the shape and thinness of the endodermal wall of the main archenteric cavity and the mesenchymal areas. The thick downwardly projecting 'peg' of notochord and its accompanying minor archenteron, passing down through the larger endoderm-lined region, is also seen. (H) Sectional level beneath that of F and G, and in a body with more evidence of directionality of pattern. The now thick-walled endoderm is invested with broad, squamous endothelial-lined cavities (*et*) reminiscent of the portal system between liver and heart regions of the normal body. A grazing section of endoderm-like the normal posterior pharynx floor (not illustrated) is also seen. Other labels as in Fig. 1. Magnification, ×63 throughout. Scale bar, 1 mm approx.

archenteron cavity passes down one side of the notochord mass and pharynx, finally entering the base of the latter so that the endoderm sequence is U-shaped. At the upper end of this minor cavity is, of course, the blastopore, which must be called posterior but is in this case surrounded by the neural area. Here is found the local appearance resembling the normal, last-recruited root of the notochord



(Cooke, 1979*b*). But the rate, and thus stage, of differentiation in the notochord mass is almost homogeneous, in contrast to the axial gradation of degree of vacuolization etc. seen along the normal body.

Beneath the pharynx is a gut region which up to stage 40 dissects as a striking radial flange or collar of thin squamous endoderm. This seems to be a radially symmetrical version of the distinct normal region just posterior to the pharynx, where outpocketing of lung occurs. The remainder of the gut, hanging downwards in gravity and finally reflexed so as to lead back up *via* the narrow crevice to the blastopore remnant, cannot be characterized. It is invested with mesoderm of lateral plate type which changes character at successive levels, visibly corresponding to local specializations seen in passing back along the normal body. There are thus areas of squamous endothelial lining reminiscent of the postcardiac region and finally, parenchymatous tissue near the borders of the cup-shaped blastocoel remnant just as in the normal liver rudiment. The solid area of tissue which in fact represents blood differentiation is near the extreme end and to one side of the 'blastocoel' edge. There is no heart.

Fig. 4. Sections through bodies with attenuated versions of the pattern restriction. (A) Grade-2 body at notochord level. Four somite segments are apparent opposite the fully differentiated, anterior notochord sector. The single focused cement gland is seen. The placode of single-layered, cuboidal epithelium at top left, though open, almost certainly represents ear vesicle. (B) Higher power view of the rear end of the body of A at a more ventral level near the blastopore. This shows an obliquely sectioned portion of the impacted, immature notochord, with small allocations of disorganized somite tissue, characteristic of the grade 2 or 1 body behind the zone of properly formed pattern. (C) Grade-1 body at notochord level. About ten proper somite segments are apparent. The mid- and hindbrain and one ear vesicle are sectioned horizontally. A zone of impacted notochord as in A is encountered between the most posterior somite seen and the blastopore. Disorganized pronephros is seen on one side. (D) Horizontal section through the middle of the optic chiasm, normally opposite the anterior boundary of the notochord in a normal stage-40 larva. (E) Section 50 μm 'ventral' to D in same larva. This passes through the significant forebrain cavity, i.e. a more 'anterior' brain region where no notochord underlies the brain. An appearance like G below is not seen until 40 μm beyond this level. (F,G) Sections through midoptic chiasm (*ch*) level, and only 40 μm ventral or 'anterior' to this, in a grade-1 pattern-restricted stage-40 larva. Note that in contrast to the normal, notochord is coextensive with brain, and the anterior section grazes the anterior end wall of the nervous system in which no forebrain formation exists in front of the optic chiasm. Other labels as in previous figures. Magnifications, (A,C) ×63, (B,D–G) ×160.

In progressively attenuated versions of the restriction, the blastopore first adopts a truly 'posterior' position and the gut adopts an elongated form under a still massive but elongating notochord and brain. It is striking that the notochord progresses to adopt a full elongation, with the normal gradation of differentiation schedule, in versions of body pattern where most of the territories that normally accompany more posterior notochord are still not included. This leads to a bunched, coiled **posterior** notochord (at a primitive stage of vacuolation – the 'stack of pennies' configuration), either embedded in the endoderm or supporting a hook-shaped projection behind the head and accompanied only by **small** masses of chaotically segmenting but undifferentiated somite (Fig. 4B). Meanwhile a variably reduced, anterior subset of somite segments is present in full form on either side of straight and fully expanded notochord. As this series of segments becomes considerable, with corresponding reduction of the zone of buckled, reflexed posterior notochord, the pattern elements of pronephros and heart return to the body, the latter starting with its posterior end as exaggerated paired portal veins and atrium only (Fig. 3). The posterior limit of proper somite segmentation and maturation is strikingly sharp in any one pattern-restricted body, and not at the blastopore (Fig. 4A,C). The blastopore is found terminating the buckled and confused zone of immature notochord and segmented material, either extremely ventrally below the gut or on a projection above and behind the brain.

In the progressively attenuated versions of the syndrome, forebrain-inducing levels of pattern are not restored until the first 20 or more somites worth of axial pattern are produced.

Discussion and conclusions

It is clear that brief exposure of the early blastula to Li^+ causes a restriction, rather than a misordering or fragmentation of the pattern formed. Continuity and order remain, but deletion from either end of a normal sequence of specifications has occurred. The limit case involves allocation of all meridians of the embryo to production of mesendodermal structure normally characterizing the posterior head region of the body and its more lateral equivalents near the first-involuted end of the mesodermal mantle. A small, but significant, territory normally anterior to this, as well as the obvious and massive posterior set, is missing. The induction of restricted levels of neural structure corresponding to mid- and hindbrain follows on.

Why is it less straightforward to discern that the Li^+ embryo has a coherent but partial body, in

comparison with *Drosophila* blastoderms from mutant eggs? Waddington (1956) and Wolpert (1971) have drawn attention to the distinction between **pattern** (i.e. regionality of epigenetic coding for body parts within cell sheets) and the mechanical processes whereby the usual architecture or **form** of a body is achieved. But they agree that an early expression of pattern is the regional specificity in mechanical contributions especially to gastrulation. In amphibians, pattern massively affects form because mesoderm designated as axial (future somite and notochord) undergoes active convergence by interdigitation of its cells. If such mesoderm possesses any anteroposterior sequence of pattern values, this is converted to a linear extension (convergent extension – Keller, 1986; Symes & Smith, 1987), probably because the cells interdigitate in a defined order. Other designations of mesoderm perform neither of these activities. In the various degrees of the Li^+ syndrome, such (notochordal) axial mesoderm as is present embraces a more or less reduced sequence of anteroposterior values, and thus tends to converge strongly, all at once. The distinctive and, at first, puzzling form of the fully restricted Li^+ body thus results from a compromise between the mechanics that would result from the expression of its true pattern alone, and the topological constraints of gastrulation whereby the root of the notochord, the closure of the archenteron cavity and the zone of recruitment of mesoderm must all be at the same place. The drawings of Fig. 5B,C with the legend should enable the gastrular morphology to be understood, in view of the restricted pattern values of mesoderm that are specified as described in the text and the other parts of this figure.

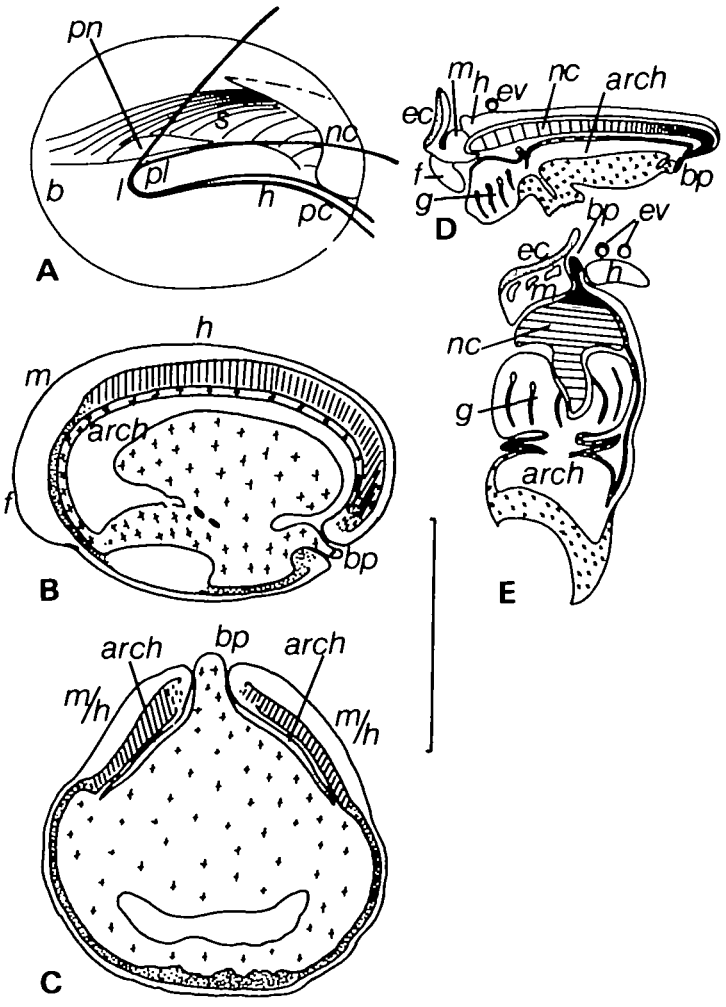


Fig. 5. The nature of the pattern restriction. (A) Fate map from the presumptive right-hand side showing the general way in which material from around the meridians of the pregastrular embryo is used in normal construction of the axial mesodermal pattern (see Cooke & Webber, 1985). The two heavy curved lines mark off restricted sectors of this map and represent subsets of mesodermal pattern that occur in the limit form and in more attenuated versions of the Li^+ syndrome. The restricted pattern is then produced, however, from mesoderm all around the embryo. (B,C) Longitudinal sections of normal neurula (sagittal plane) and of synchronous form fully radialized after blastula lithium treatment (compare with Cooke & Smith, 1987, fig. 7 for u.v.-induced nonaxial development). Axial (notochordal) mesoderm is shown hatched, other mesoderm stippled, and endoderm indicated by crosses. Differences in thickness between axial and other mesoderm, and between neuralized and epidermal ectoderm, are somewhat exaggerated in relation to the early neurula stage 14 actually dissected. In C, the cavity of the original blastocoel remnant, pushed ventrally and free of mesoderm in the normal case, is obliterated by contact from the endoderm onto its epidermal wall whose inner face appears to have been ectopically mesodermalized. A new intraendodermal cavity forms to conserve the volume of the embryo (see also embryos treated with soluble mesoderm-inducing factor – Cooke *et al.* 1987). The embryos are drawn as they orientate in gravity, as the annular, shallow archenteron of form C causes its blastopore to face upwards as well as being surrounded by the neuralized and notochordal areas. There is later a transformation to give an archenteric passage by-passing a notochordal mass, because of the latter's demand for convergence – minimization of surface area in relation to cell number. (D,E) Exploded diagrams of notochord, induced neural and endodermal (gut) structure in the normal and the limit pattern-restricted larval body. Since gastrulation effectively turns the presumptive gut and mesoderm inside out, reversing its polarity, the normal body is drawn mirror-imaged with respect to A. Labelling of structures is as in previous figures, with addition of *pc*, prechordal region, *h*, heart-forming region, *b*, blood-forming region and *f*, *m*, *h*, fore- mid- and hindbrain regions of neuralized ectoderm and CNS. Scale bar, 1 mm approx. for drawings of A–C.

Fig. 5A,D,E show the pattern restriction in terms of the map for normal origin of mesodermal structures at gastrulation and as schematic exploded diagrams of larval structure. The displayed layout of somite numbers and borders and other structures on Fig. 5A does not imply a belief in a mosaic of specifications at such early stages. There is appreciable cell mixing between the stage of Li^+ application and the final specification of mesodermal territories. But, in our hands, lineage labelling does reveal a regularity to the way in which material is normally incorporated into the mediolateral and anteroposterior sequences of mesodermal body structure, that is aptly described by the layout given. Due to some cumulative physiological effect of the Li^+, at whatever subsequent stage the pattern values in mesoderm do actually stabilize, values within the more or less restricted spectra shown by the boundaries drawn on Fig. 5A are adopted throughout. Following progressively later onset or lower concentrations of experienced Li^+, the permitted subset of mesoderm is expanded towards completion in posterior and ultimately anterior directions.

Although the restriction is in general more an anteroposterior than a dorsoventral one, the normal 'ventral' region for blood specification lies so far outside it that the undoubted presence of this tissue in full Li^+ forms needs further explanation. Two kinds of such explanation are possible, one having to do with inhibitory signals between specific pattern parts and the other proposing that Li^+ causes ectopic mesoderm to be produced in addition to respecifying the character of the expected mesoderm. In terms of tissue distance, the blood arises far from the massive peg of notochord in the radial dorsoanterior embryo, and there is little or none of the normal complement of somite. Thus if dorsal axial mesoderm, as generally believed (Slack & Forman, 1980; Cooke & Smith, 1987), or even somite specifically, limits the spatial domain of blood formation by a transcellular signal, such signalling may be inadequate in the abnormal geometry of the present embryos. Blood could then arise, even when we would not otherwise expect it, in tissue that would in any case have been mesoderm. Alternatively, tissue of the blastocoel roof, which lies beyond the normal limit for specification of mesoderm by the vegetal inducing signals, may in these embryos become mesodermal, if the Li^+ effect modulates the threshhold levels for responses to those signals which in fact reach throughout the animal cap. We might expect any such ectopic mesoderm to include blood, as the traditionally 'ventral' or low-threshhold mesodermal specification. This hypothesis is mentioned here because related work in progress, characterizing interactions between Li^+ and the responses *in vitro* of animal pole tissue to mesoderm inducers, indicate that the blastocoel roof tissue is indeed mesodermal after Li^+ treatment of blastulae. Fix-dissection of stage-14 neurulae (see Fig. 5B,C) reveals that what would normally be the epidermal covering of the blastocoel remnant is already behaving as ectopic mesoderm in terms of cell contact behaviour. This second 'ectopic' explanation for the presence of blood near the blastocoel floor, in the pattern of Li^+ embryos, is thus favoured at the present time.

Lewis & Wolpert (1971) proposed, with their principle of nonequivalence in development, that embryonic tissue becomes somehow coded as to body position, in a system of regionalization that is independent of the differentiations finally recognized by the cytologist and histologist. The full and the attenuated versions of the Li^+ syndrome offer much evidence that nonequivalence, now well attested in insect development, operates in the embryogenesis of vertebrates, and that Li^+ here acts largely by restricting the graded sequence of body positional codings in at least mesoderm. Thus, in the limit form, the entirety of notochord differentiates synchronously on the relatively precocious schedule normal to its anteriormost region, where characteristically few somite cells are also allocated in the normal body. The view that sees mesodermal pattern mainly in terms of inductive relations between visible tissue types (e.g. Slack & Forman, 1980) would by itself predict that mesoderms possessing massive notochord would be overloaded also with somite muscle. The attenuated syndrome is represented by an approach towards the normal rostrocaudal graduation of notochord differentiation schedule and form, as well as the proper placement and segmentation of distinctively anterior, i.e. early-forming, subsets of the somites. Conversely, partial u.v. impairment of pattern from earliest stages allows only distinctively posterior, late-forming subsets of segments to develop.

The coiled posterior notochord of low-grade Li^+ axes, without proper somite bodies, is understandable because the entire notochord sequence is specified at relatively dorsal and anterior locations. The key seems to be the time of recruitment in the preorganized sequence of gastrulation. For the entire notochord, this is relatively early (Cooke, 1979*b*; Keller, 1976), whereas most of the material for the massive posterior somite bodies is specified as late-recruited, over a much longer period. As this is from a part of the normal gastrular specification map that is excluded by even the attenuated Li^+ restriction, these forms literally have no mesoderm remaining to be allocated to posterior somite production.

As would be predicted from previous observations (e.g. Cooke, 1986) the rates of progression to the point of gastrulation around the embryo, and the

character of the involutional activity when it occurs, are diagnostic of the pattern values of material as seen in the formed body. In the full Li^+ syndrome, blastoporal activity first appears in a full circle near the time of the dorsal onset of activity in controls. The lip then has entirely the character of that near the dorsoanterior meridian of normal embryos, which is accompanying archenteron invagination as well as mesoderm formation. This observation fits well the idea that the radially symmetrical system represents a narrow belt of the relatively anterior position values, normally lying lowest in the marginal zone and among the first to be recruited into involution (Keller, 1976). The very rapid but symmetrical progress to lip closure (stage 13 equivalent) similarly fits with the supposition that a large set of the posterior pattern values, which normally extend the process of gastrulation in time, is missing. One possible explanation of the distinctive loss of prechordal head structure and induced nervous system (Fig. 4) may lie in the observations from fix-dissection of neurulae (Fig. 5B,C). If the original blastocoel roof is now of ectopically mesodermal character, the normal spread upon it of the thin prechordal mesoderm may be inhibited. Such a spreading tendency, though endogenous to this mesodermal region (Keller, 1986), may need to be expressed in order for proper head organization and induction to occur.

Physiological mode of action

In our hands, Li^+ will not act as a mesodermal inducer for isolated, naïve ectoderm, even though it may modulate the spectrum of responses to actual mesoderm induction in a way that goes far to explain the whole embryo syndrome. The definable beginning and end of a sensitive period for blastular Li^+ treatment do not enable us to define the time of the process that the ion is altering. We do not know its rate of release from the relevant physiological compartment of the embryo after removal from the environment, or the length of time for which it must act in order to bring about its effect. The blastocoel may complicate the process by acting as an inner store, but the data of Breckenridge *et al.* suggest at least that build-up after application is reasonably rapid. The end of the sensitive phase is coincidental with only the beginning of a midblastular transition to zygotic transcriptional activity, suggesting that Li^+ affects a process in connection with body position values that precedes their recording at the chromatin or 'genetic read-out' level.

There has been recent evidence that Li^+ acts on the pathway of protein phosphorylation and second messenger production that involves protein kinase C and inositol lipid metabolism, and this might seem as good a candidate site as any for its role in distorting positional specification. It should therefore be mentioned that a rather thorough study of the effects upon whole embryos of the phorbol ester TPA (12-0-tetradecanoyl phorbol-13-acetate), which artificially activates protein kinases C, indicated no effects upon whole body pattern formation (though see Davids *et al.* 1987). Prolonged or acute culture at stages between the fertilized egg and gastrula, at concentrations that obviously penetrated cells within seconds and caused massive activation of the subsurface contractile apparatus while just failing to cause cytolysis, were always without any effect on pattern proportions or polarity in surviving individuals. We have not, however, tested other molecules that might be rate-limiting on this pathway, such as inositol derivatives, nor have we tested for specific ability of such treatments to rescue from, or to exacerbate, the Li^+ syndrome. The above negative findings are not therefore strong evidence against involvement of the pathway in this morphogenetic effect and thus, by implication, in responses of competent tissue to mesoderm inducing stimuli.

Note added in proof

The recent study by Condie & Harland (*Development* **101**, 93–106) dealing with early expression of a homeobox-containing gene in *Xenopus*, has utilized the Li^+ embryo as a differential screen in the way proposed in the present paper.

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