Development 122, 371-379 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV0047



# The alignment of the axis of asymmetry in regenerating protoplasts of the moss, *Ceratodon purpureus,* is determined independently of axis polarity

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## SUMMARY

***Ceratodon* protoplasts regenerate by polar outgrowth to form cell filaments. The kinetics of regeneration show that some cellular event has to be completed before regeneration can be initiated. The development of the regeneration axis is strongly influenced by light, with axis alignment and axis polarity being fixed independently. We define axis alignment as the relationship of the regeneration axis to the incident light, independent of polarity. Thus protoplasts regenerating directly towards, or directly away from the light source are defined as being similarly aligned but with opposite polarity. Protoplasts that regenerate in unidirectional red light form axes that are aligned parallel to the light direction, with about 70% being polarised towards the light and about 30% away. In unidirectional blue or white light, almost all protoplasts regenerate towards the light but axis alignment is determined less stringently. Re-orientation of protoplasts regenerating in unidirectional light shows that axis alignment is fixed between 8 and 9 hours before protoplasts regenerate and that axis polarity is fixed later. When protoplasts are removed from directional light to either non-directional light or to darkness, regeneration axes continue to be aligned by the earlier directional stimulus for at least 24 hours. Thus although axis alignment is fixed only about 8 hours before regeneration, in the absence of contradictory information about directionality in the light environment, protoplasts retain a memory of light direction for much longer. However, both reorientation and removal from a directional light field have profound effects on axis polarity; the pattern observed in undisturbed protoplasts being lost. To account for these observations, we propose that separate gradients are established independently to determine the alignment and polarity of the regeneration axis respectively. The alignment gradient is established rapidly and is steeper in red than in blue or white light, the polarity gradient is established slowly and is steeper in white or blue light than in red. These studies will now allow a genetic dissection of these processes in moss.**

Key words: asymmetry, moss, polarity, protoplast, regeneration, axis formation, *Ceratodon purpureus*

## INTRODUCTION

The generation of asymmetry is fundamental to development. In many biological systems, the programming of asymmetry is dependent on pre-existing asymmetry (Chant and Pringle, 1991; Horvitz and Herskowitz, 1992; Tautz, 1992). In other systems, particularly in plants and sedentary animals, the induction of asymmetry is related to development in a non-uniform environment. For example, *Fucus* zygotes are symmetrical until 16 hours after fertilisation when a rhizoid develops by polar outgrowth. The axis of rhizoid outgrowth can be aligned by the imposition of a variety of gradients on the zygote, the most studied being light. The alignment of the axis with respect to a light gradient can be set by a 1- to 2-hour light treatment given between 4 and 10 hours following fertilisation. The alignment of the axis is labile during this period, but between 10 and 12 hours postfertilisation the alignment of the axis becomes fixed according to the light direction most recently perceived by the zygote. Polar outgrowth of the rhizoid occurs on this fixed axis and always occurs away from the source of light (Goodner and Quatrano, 1993; Fowler and Quatrano, 1995).

We were interested in determining whether the generation of asymmetry in a non-uniform environment by another cell type demonstrated the same properties as *Fucus* zygotes, i.e. with separate processes of axis formation and fixation and with the generation of polarity along an axis aligned by an externally imposed gradient. We chose the regenerating protoplast of the moss *Ceratodon purpureus*, not only because the development of the regeneration axis of *Ceratodon* protoplasts is influenced by an external light gradient, but also because the moss system will allow a genetic approach to dissect the mechanisms involved in the establishment of asymmetry (Knight and Cove, 1989; Cove, 1992; Knight, 1994).

Protoplasts of the mosses, *Ceratodon purpureus* and *Physcomitrella patens*, released by the enzymatic removal of

the cell wall from protonemal tissue, appear to be symmetrical spheres. Isolated protoplasts regenerate directly to give rise to protonemal filaments. Like *Fucus* zygotes, moss protoplasts become asymmetrical by developing a polar outgrowth, which after the first cell division, forms the apical cell of a filament (Fig. 1A). The light requirements for regeneration of protoplasts of *P. patens* have been studied previously (Jenkins and Cove, 1983a). Asymmetrical regeneration in this species only occurs in high light levels. Studies of *P. patens* have shown that the axis of regeneration can be oriented by regeneration in a directional light field (Burgess and Linstead, 1981). We report here more detailed studies of the role of light in determining polar axis formation during protoplast regeneration in *C. purpureus*, a species chosen because its protoplasts do not require light for regeneration, allowing the effects of a wider range of light conditions to be investigated in the study of axis determination. Our studies reveal that the generation of asymmetry involves two separable steps: (1) about 8-9 hours before polar outgrowth, the alignment of the regeneration axis is fixed, and (2) polarity along the fixed axis is then determined.

## MATERIALS AND METHODS

### *Ceratodon purpureus* wild-type strain

The wild-type strain (WT3) used was originally isolated from a single spore (Hartmann et al., 1983), and has been propagated asexually since isolation.

### Protoplast isolation

Protoplasts were isolated from tissue that had been grown on cellophane overlying solid medium. The medium used was based on a modified Knop's medium (Ashton and Cove, 1977; Knight et al., 1988) but contained 10 mM $KNO_3$ plus 10 mM $CaCl_2$ instead of 5 mM $Ca(NO_3)_2$, i.e. twice the level of calcium. The medium was supplemented with 5 mM ammonium tartrate. The procedures used for protoplast isolation were essentially similar to those used for *P. patens* (Grimsley et al., 1977) with the following minor modifications. Tissue was not incubated in 8% mannitol before the addition of enzyme (Driselase) as this was found not to be necessary. Enzymatic digestion was carried out for 20 minutes at 25°C with only occasional gentle agitation. After enzymatic digestion, protoplasts were separated from tissue debris by straining through sterile nylon cloth having a pore size of 70 μm × 70 μm.

### Conditions for protoplast regeneration

Protoplasts were regenerated embedded in a thin layer of solid medium on cellophane overlying a deep layer of solid medium, following the method of Grimsley et al. (1977). For most experiments, 50 mm diameter Petri dishes were used and these required only 700 μl of the overlay medium containing protoplasts to cover the cellophane. Protoplasts were regenerated in the same modified Knop's medium used to obtain tissue, as described above, except that sucrose was included at 0.5% (w/v) and mannitol at 6% (w/v) was added as an osmotic buffer.

Protoplasts were regenerated at 25°C. For unidirectional light treatments, Petri dishes were placed into individual light-tight boxes from which one side was removed. Except where stated otherwise, the Petri dishes were incubated horizontally, and illuminated from the side so that the light direction was at right angles to gravity. The light intensity at the centre of Petri dishes incubated in these boxes was estimated to be about 10% of that at the illuminated edge. Light intensities were routinely measured at the position to be occupied by the open side of the box and the values reduced to allow for the observed light attenuation. Monochromatic light was obtained using interference filters (DAL, Schott, Mainz, Germany) with projectors having quartz-halogen bulbs. The levels of monochromatic light used were the highest available to us. White light was provided by fluorescent tubes (Philips MCFE white). Omnidirectional white light was given to Petri dishes placed horizontally on white paper.

### Light treatments

Except where stated otherwise, the following light treatments were used. Unidirectional red: wavelength 665 nm; intensity 1.5 μmol quanta/$m^2$/second. Unidirectional blue: wavelength 437 nm; intensity 400 nmol quanta/$m^2$/second. Unidirectional white: intensity 7.5 μmol quanta/$m^2$/second. Omnidirectional white: intensity 75 μmol quanta/$m^2$/second.

### Scoring regeneration

Protoplasts were scored as having regenerated when they could be seen to be asymmetrical (Fig. 1A, stage (ii) and beyond). Axis alignment and polarity was scored by aligning the regeneration axis to an array of parallel lines on a graticule in a microscope eyepiece. The eyepiece was coupled by gears to a 10-turn variable resistor, allowing readings to be inputted direct to a computer by way of an analogue/digital interface. This procedure allowed alignment to be determined consistently to an accuracy of at least ±2°. Most protoplasts showed unipolar regeneration. Where bipolar regeneration occurred and the two outgrowths were unequal, the longer of the two outgrowths was scored. Protoplasts having two equal outgrowths (less than 2% of total) were not scored. Unidirectional red light intensities greater than 1.5 μmol quanta/$m^2$/second were achieved by observing protoplasts near to the open edge of the box in which they were incubated. For all other treatments, only protoplasts regenerating in the central 8 mm × 8 mm area of the Petri dish were scored. This region was scanned systematically until either all the protoplasts within it or 200 protoplast had been scored. Protoplasts were sampled from at least two Petri dishes for each treatment.

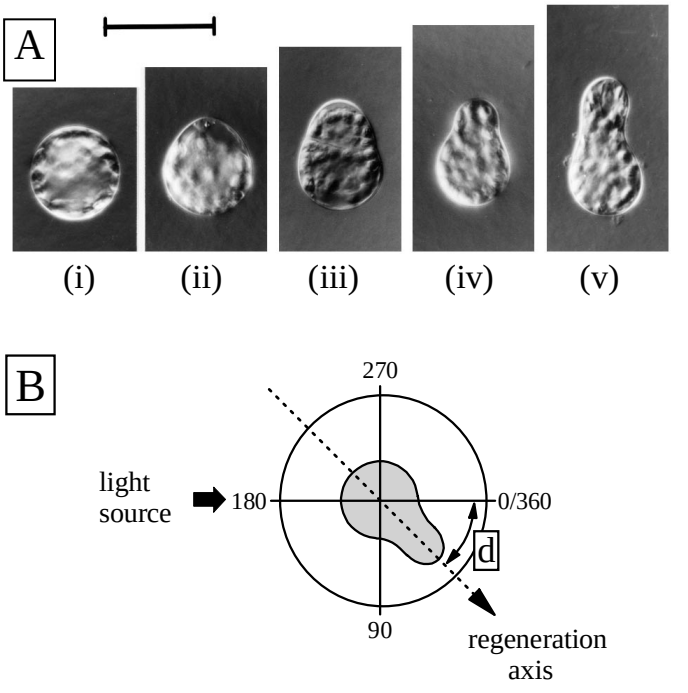


**Fig. 1.** (A) Regenerating moss protoplasts. Scale bar, 40 μm. (B) Measurement of alignment and polarity of regeneration axis. (For explanation see Materials and methods section.)

### Data collection and processing

Data for the axis of each regenerated protoplast was stored as the angle between the regeneration axis and the direction of the light source (Fig. 1B, angle d). Protoplasts regenerating directly towards the light source would have a value of 180°, those regenerating directly away, a value of 0°. Data for complete samples were displayed as circular histograms. The extent to which the regeneration axis was aligned with the light direction was analysed independently from the polarity of the axis (i.e. whether outgrowth was towards or away from the light source). Numerical values for orientation and polarity were computed as follows.

#### Alignment

The mean angle of deviation from the light direction was computed. Note, for example, that protoplast axes with angles of 45°, 135°, 225° and 315° will all have the same deviation from the light direction, i.e. 45°, and so axis alignment is independent of polarity (and handedness).

#### Polarity

The proportion of protoplasts in a sample regenerating towards the light source, (i.e. the proportion of protoplast axes with values between 91° and 270°), was used as a measure of axis polarity.

The deduced distribution of axis alignment and polarity for a cohort of protoplasts regenerating between two sampling times was obtained by the following procedure. The distribution for the earlier time was weighted by the ratio of protoplast regenerated at the earlier time to that at the later, and was then subtracted from the distribution at the later time. The resulting deduced distribution for protoplasts regenerating between the two times was then normalised to give percentage values. Thus, for example, to generate the values given for protoplasts regenerating in unidirectional red light between 15 and 20 hours (see Table 2A), the values for 15 hours were reduced by 6/12, the ratio of regeneration at 15 hours (6%) and 20 hours (12%), and then subtracted from the values at 20 hours. Where the value generated was less than 0, a value of 0 was recorded. The total of all the deduced values was then determined and used to normalise the deduced distribution so that it totalled 100%.

## RESULTS AND DISCUSSION

### The kinetics of protoplast regeneration

Protoplast regeneration was investigated in different light conditions. Protoplasts which showed any degree of asymmetry were scored as having regenerated. The kinetics of regeneration in blue light, red light and in darkness are shown in Fig. 2.

The kinetics of protoplast regeneration in monochromatic red and blue light and in darkness show essentially similar forms, with a lag phase before any regeneration is observed, followed by a linear increase in the number of protoplast that have regenerated. Both the length of the lag phase and the slope of the regression vary with light conditions and the two are correlated.

The kinetics observed are not those expected if differences in regeneration times between protoplasts were caused by random variation since these would generate a sigmoid cumulative distribution curve corresponding to a normal distribution of regeneration times. Instead, the observed kinetics suggest that a protoplast must wait for some event to be completed before regeneration is possible. The variation in the lag time and in the observed slopes in different light conditions is probably accounted for, at least in part, by differences in photosynthetic activity under the conditions investigated. In all cases, sucrose was present in the regeneration medium but in the related moss *P. patens*, photosynthetic mutants cannot be restored to the growth rate of non-mutant strains by supplementation with sucrose (Long, 1987). It is also possible that light plays a more direct role in regulating the kinetics of regeneration. The tissue from which protoplasts are isolated consists largely of chloronemal cells. Although chloronemal cells of *C. pupureus* can divide in darkness, those from *P. patens* have a developmental requirement for light in order to divide (Jenkins and Cove, 1983b). Detailed studies of cell cycle times in different light conditions have not been carried out in *C. purpureus* but it is possible that the differences in the kinetics of regeneration observed are due to a requirement for a protoplast to have reached a particular stage in the cell cycle before regeneration can be initiated.

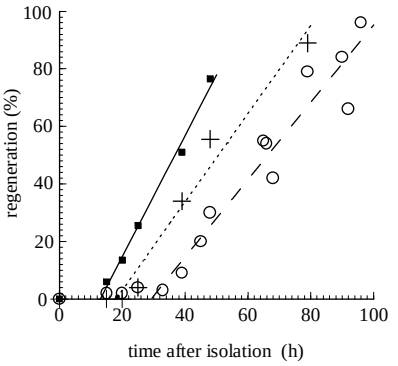


**Fig. 2.** Kinetics of protoplast regeneration. Red light, solid symbols and unbroken line; blue light, crosses and short-dashed line; darkness, open circles and long-dashed line. Each line is a regression of all data points with a value greater than 5%. Light conditions and procedures for protoplast isolation and regeneration are described in the Materials and methods section.

### Alignment and polarity of protoplast regeneration axes, 48 hours after plating

The alignment and polarity of the regeneration axes of populations of protoplasts that had regenerated under a range of light conditions were scored as described in the methods section. Details of the experimental conditions, and of axis orientation and polarity are given in Table 1. The distributions obtained for some of these conditions are shown as circular histograms in Fig. 3.

In all cases, the orientation of protoplast regeneration axes was scored normal to the agar surface. Control platings in darkness where the plates were incubated horizontally and gravity was therefore at right angles to the plane in which protoplasts regenerated (see Table 1 and Fig. 3E) showed a random distribution of regeneration axes (a random distribution generates a mean deviation of regeneration axes from any arbitrarily chosen axis of 45° and a figure of 50% for axes positive to any arbitrarily chosen direction). When protoplasts were regenerated in darkness in Petri dishes which were vertical so that gravity was effective in the plane in which the orientations of regeneration axes were scored (see Table 1 and Fig. 3F), a slight effect of gravity was detectable. In all other experiments, Petri dishes were incubated horizontally so that the effect of gravity was confounded.

Whereas rhizoid outgrowth in *Fucus* is always away from the

**Table 1. Alignment and polarity of protoplast regeneration axes in different light conditions**

| Light and growth conditions | Light intensity (μmol quanta/$m^2$/second) | Sample size | Axis alignment (mean angle of deviation in degrees) | Axis polarity (% axes towards light source) |
|---|---|---|---|---|
| Unidirectional white light | 30 | 396 | 35 | 89 |
| | 7.5 | 800 | 38 | 91 |
| | 5 | 123 | 39 | 91 |
| Unidirectional red light | 10 | 346 | 24 | 71 |
| | 2.0 | 290 | 19 | 58 |
| | 1.5 | 1017 | 14 | 69 |
| | 0.3 | 227 | 21 | 84 |
| Unidirectional blue light | 0.4 | 171 | 34 | 84 |
| Omnidirectional white light | 75 | 600 | 45* | 50* |
| Darkness (Petri dishes horizontal) | - | 123 | 45* | 50* |
| Darkness (Petri dishes vertical) | - | 600 | 44† | 41† |

All treatments were scored 48 hours after isolation with the exception of the darkness (Petri dishes vertical) treatment which was scored 72 hours after isolation.

*Since no directional light was given for these treatments, alignment and polarity were scored with respect to an arbitrarily chosen direction.

†Alignment and polarity were scored with respect to the direction of the earth, thus 41% polarity represents a slight bias in axes oriented away from gravity.

orienting light gradient, i.e. polarity is invariant, we found that the alignment and polarity of protoplast regeneration axis are not tightly coupled in *C. purpureus.* The distributions of axis alignment and polarity observed in different light conditions are therefore best discussed if alignment and polarity are considered separately. Preliminary experiments using a range of intensities of unidirectional white and monochromatic red light (see Table 1) showed that axis alignment was more sharply determined in monochromatic red light than in white and that axis polarity was more sharply determined in white light than in red. Within the range of intensities of red or white that was studied, light intensity did not have a marked effect on either axis alignment or polarity. Further experiments, comparing a single intensity of red light (1.5 μmol quanta/$m^2$/second) and white light (7.5 μmol quanta/$m^2$/second) with monochromatic blue light (0.4 μmol quanta/$m^2$/second), showed that blue light had a similar effect to white light on both axis alignment (mean angle of deviation 38° in white, and 34° in blue, c.f. 14° in red) and on axis polarity (91% of protoplast regeneration axes are oriented towards the light source in white light, 84% in blue, c.f. 69% in red).

## Kinetic studies of the determination of the alignment and polarity of the regeneration axis

To determine whether axis alignment and polarity were affected by the time at which protoplast regeneration occurred, detailed kinetic studies were carried out to investigate protoplast regeneration throughout the period in which the majority of protoplast regenerated.

Table 2A and Fig. 4A summarise the results of experiments carried out in unidirectional red light. Similar experiments were carried out for protoplasts regenerating in unidirectional blue and white light and the results of these experiments are summarised in Table 2B and 2C. These experiments established that the distribution of axis alignment and polarity was not affected in any of these light conditions, by the time at which protoplast regeneration occurred.

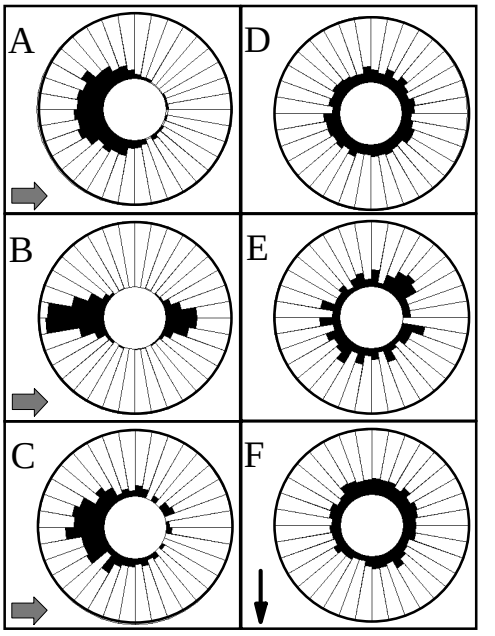


**Fig. 3.** Circular histograms of distribution of axis alignment and orientation in unidirectional red light, blue light, white light, in omnidirectional white light and in darkness. The histograms show the distribution of the regeneration axes, plotted at 10° intervals. The shaded areas are proportional to the percentage of protoplast regenerating in any 10° sector. Shading to the outer circle would represent a value of 25%. (A) Scored after 48 hours regeneration in unidirectional white light. In A,B and C the arrow to the left indicates the light direction. (B) Scored after 48 hours regeneration in unidirectional red light. (C) Scored after 48 hours regeneration in unidirectional blue light. (D) Scored after 48 hours regeneration in omnidirectional white light. (E) Scored after 48 hours regeneration in darkness, Petri dishes horizontal, gravity perpendicular to plane scored. (F) Scored after 72 hours regeneration in darkness, Petri dishes vertical. The arrow to the left points down towards the earth. Light conditions and procedures for protoplast isolation and regeneration and for scoring the distribution of regeneration axes are described in the Materials and methods section.

## The effect of reorientation on the alignment and polarity of the regeneration axis of protoplasts regenerating in unidirectional light

Regenerating protoplasts were reoriented with respect to the light direction. Table 3A and Fig. 4B summarise experiments in which protoplasts regenerating in unidirectional red light, were reoriented by 90°, 15 hours after isolation. These experiments show that reorientation affects the alignment and polarity of the regeneration axis differently. Regeneration axes continue to be oriented by the first light direction for some time after reorientation. There is little detectable effect on alignment in the first five hours following reorientation. Protoplasts regenerating between 10 and 24 hours following reorientation are clearly preferentially aligned parallel to the second light direction but axis alignment is less precisely determined than in protoplast regenerating undisturbed in red light. The switch in axis alignment from the first to the second light direction therefore occurs between 5 and 10 hours after reorientation.

**Table 2. Alignment and polarity of regeneration axes of protoplasts regenerating undisturbed in unidirectional light***

| Interval (hours after isolation)† | Regeneration (%) | Axis alignment (mean angle of deviation in degrees) | Axis polarity (% axes towards light source) |
|---|---|---|---|
| A. red light | | | |
| 0 to 15 | 6 | 15 | 68 |
| 15 to 20 | 6 | 22 | 79 |
| 20 to 25 | 8 | 17 | 74 |
| 25 to 39 | 29 | 13 | 72 |
| 39 to 48 | 21 | 14 | 77 |
| B. blue light | | | |
| 0 to 20 | 0 | - | - |
| 20 to 25 | 5 | 30 | 92 |
| 25 to 39 | 22 | 30 | 92 |
| 39 to 48 | 29 | 39 | 80 |
| 48 to 72 | 36 | 38 | 87 |
| C. white light | | | |
| 0 to 20 | 0 | - | - |
| 20 to 25 | 4 | 43 | 84 |
| 25 to 39 | 19 | 40 | 93 |
| 39 to 48 | 24 | 45 | 84 |
| 48 to 72 | 37 | 32 | 95 |

*For details of light treatments, see Materials and methods section

†Data for a time interval were computed by subtracting the distribution at the beginning of the interval from that at the end (see Materials and methods section).

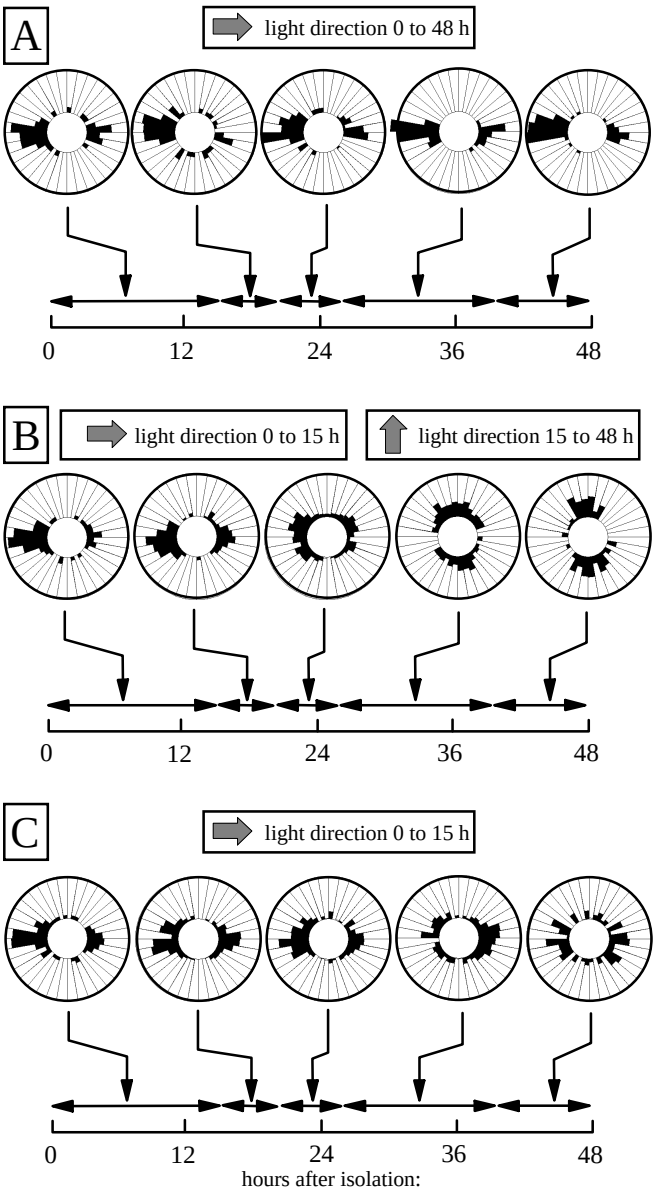


**Fig. 4.** Kinetics of axis determination in regenerating protoplasts. (A) Protoplast regenerating undisturbed in unidirectional red light. (B) Protoplast regenerating in unidirectional red light, reoriented by 90° with respect to the light direction, 15 hours after isolation. (C) Protoplasts, cultured in unidirectional red light for 15 hours and then transferred to omnidirectional white light. The circular histograms show the distribution of regeneration axes in protoplasts in the time intervals indicated. For further details see text and legend to Fig. 3.

Reorientation also affects the determination of the polarity of the regeneration axis. Protoplast regenerating undisturbed in unidirectional red light, consistently show a pattern of axis polarity with about 70% of axes polarised towards the light direction and 30% away from it. Reorientation disturbs this polarity bias so that more or less equal numbers of axes are polarised towards and away from the new light direction. Reorientation of protoplasts therefore affects the alignment of the regeneration axis independently of its polarity. Protoplasts realign their axes to the new light direction but are unable to polarise the axes correctly following reorientation.

In order to establish more precisely when axis alignment and polarity were determined, experiments were carried out in which populations of protoplasts, incubated in unidirectional red light, were reoriented at different times following isolation. The distribution of regeneration axes orientations was scored 48 hours after isolation and the results of these experiments are given in summary form in Table 4. Regeneration axis distributions were simplified to give four 90° quadrants (see Table 4 for details). A model was investigated in which it was assumed that axis alignment was fixed before axis polarity. Protoplasts that had fixed the alignment of their axis before reorientation, were assumed to align their axes parallel to the original light direction, those that fixed axis alignment after reorientation were assumed to align their axes parallel to the second light direction. Protoplasts that fixed axis polarity before reorientation were assumed to show a distribution of 68% in quadrant 1 (towards the first light direction) and 2% in both quadrants 2 and 4 (lateral to the first light direction). This distribution is the mean of all experiments for protoplasts regenerating undisturbed in unidirectional red light of the same intensity (c.f. Fig. 3B). Those protoplasts that fixed axis polarity after reorientation were assumed to show a distribution of 50% in quadrant 2 (towards the second light direction), and 5% in both quadrants 1 and 3 (lateral to second light direction). This distribution was chosen as approximating to the mean of observed distributions. The kinetics of regeneration was modelled using the regression line for red light shown in Fig. 2. Using these parameters, the time of fixation of axis alignment and polarity was varied by half hour increments to obtain a best fit of the experimental data by minimising the sum of squares of the differences between the modelled distribution and the experi-

**Table 3. Effect on alignment and polarity of regeneration axes of protoplasts regenerating in unidirectional light*, of reorientation by 90° 15 hours after isolation**

| Interval (hours after isolation)§ | Regeneration (%) | Relative to light direction 1† | | Relative to light direction 2‡ | |
|---|---|---|---|---|---|
| | | Axis alignment (mean angle of deviation degrees) | Axis polarity (% axes towards light source) | Axis alignment (mean angle of deviation in degrees) | Axis polarity (% axes towards light source) |
| A. Red light | | | | | |
| 0 to 15 | 6 | 16 | 76 | - | - |
| 15 to 20 | 8 | 19 | 63 | 71 | 36 |
| 20 to 25 | 17 | 31 | 63 | 59 | 60 |
| 25 to 39 | 22 | 57 | 50 | 33 | 62 |
| 39 to 48 | 23 | 65 | 52 | 25 | 46 |
| B. Blue light | | | | | |
| 0 to 20 | 0 | - | - | - | - |
| 20 to 25 | 3 | 40 | 82 | 50 | 60 |
| 25 to 39 | 39 | 45 | 71 | 45 | 66 |
| 39 to 48 | 14 | 40 | 53 | 34 | 90 |
| 48 to 72 | 32 | 55 | 60 | 48 | 61 |
| C. White light | | | | | |
| 0 to 15 | 0 | - | - | - | - |
| 15 to 20 | 1 | 46 | 89 | 44 | 67 |
| 20 to 25 | 4 | 45 | 89 | 45 | 70 |
| 25 to 39 | 43 | 49 | 77 | 41 | 81 |
| 39 to 48 | 32 | 49 | 68 | 41 | 87 |

*For details of light treatments, see Materials and methods section.
†Light direction 1: 0 to 15 hours.
‡Light direction 2: 15 hours onwards.
§Data for a time interval were computed by subtracting the distribution at the beginning of the interval from that at the end (see Materials and methods section).

mental data. The best fit is obtained when axis alignment is fixed 8.5 hours before regeneration and axis polarity is fixed 7 hours before regeneration. Table 4 summarises the experimental data and the modelled distributions obtained using these figures. It is likely that this model could be refined further but it is doubtful whether the present data is extensive enough to warrant this. Varying the distribution parameters shows that the figure for the time of fixation of axis alignment is robust but that the time of fixation of axis polarity is less robust and may need to be revised down. The data therefore clearly support the conclusion that the alignment of the regeneration axis is fixed between 8 and 9 hours before a protoplast regenerates. It is probable that axis polarity is fixed later.

Reorientation experiments were also carried out in blue and white light and the results of these are summarised in Table 3. The same trends can be seen when protoplasts are reoriented in either blue (Table 3B) or white (Table 3C) light but because axis alignment is less stringently determined under these light conditions, the data do not allow the same type of detailed model to be fitted. The qualitative data suggest that axis alignment is fixed at about the same time in white light as in red and somewhat earlier in blue light. The effects on axis polarity of reorientation in red, blue and white light are also summarised in Table 3. In contrast to red light, reorientation in white light has little effect on the polarity of the regeneration axis while reorientation in blue light may have a smaller effect.

**Table 4. Experimental results and modelled predictions of reorientation in unidirectional red light***

| Time re-oriented | Time scored | | Quadrant† 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|
| A. Protoplasts reoriented at varying times after plating, scored at 48 hours | | | | | | |
| 4 | 48 | observed | 5 | 51 | 8 | 36 |
| | | *modelled* | *5* | *50* | *5* | *40* |
| 8 | 48 | observed | 12 | 53 | 5 | 30 |
| | | *modelled* | *11* | *45* | *8* | *36* |
| 10 | 48 | observed | 15 | 50 | 8 | 27 |
| | | *modelled* | *14* | *43* | *9* | *34* |
| 12 | 48 | observed | 12 | 53 | 9 | 26 |
| | | *modelled* | *18* | *40* | *11* | *32* |
| 15 | 48 | observed | 25 | 32 | 15 | 28 |
| | | *modelled* | *23* | *36* | *13* | *29* |
| 17 | 48 | observed | 28 | 33 | 12 | 27 |
| | | *modelled* | *27* | *33* | *14* | *26* |
| 20 | 48 | observed | 27 | 33 | 13 | 27 |
| | | *modelled* | *32* | *29* | *16* | *23* |
| B. Protoplasts reoriented at 15 hours after plating, scored at varying times | | | | | | |
| 15 | 15 | observed | 71 | 2 | 20 | 6 |
| | | *modelled* | *67* | *2* | *29* | *2* |
| 15 | 20 | observed | 59 | 4 | 33 | 4 |
| | | *modelled* | *67* | *2* | *29* | *2* |
| 15 | 25 | observed | 57 | 8 | 27 | 7 |
| | | *modelled* | *52* | *9* | *31* | *8* |
| 15 | 39 | observed | 34 | 26 | 20 | 20 |
| | | *modelled* | *29* | *31* | *15* | *25* |
| 15 | 48 | observed | 21 | 35 | 12 | 32 |
| | | *modelled* | *23* | *36* | *13* | *29* |

Values given in the quadrant columns are percentages of axes oriented in the relevant quadrant.
*For details of light treatments, see Materials and methods section.
†Quadrant definitions (angles refer to orientation with respect to first light direction - see figure 1): quadrant 1, towards first light direction ±45° (136° to 225°); quadrant 2, towards second light direction ±45° (226° to 315°); quadrant 3, away from first light direction ±45° (316° to 360° and 1 to 45°); quadrant 4, away from second light direction ±45° (46° to 135°) .

## The effect of removal of protoplasts from unidirectional light on the alignment and polarity of the regeneration axis

Protoplasts were incubated following isolation in unidirectional light and then transferred either to omni-directional white light or to darkness.

The result of transferring protoplasts after 15 hours in unidirectional red light to omni-directional white light are shown in Table 5A and Fig. 4C. It will be seen that axis alignment by the cohort of protoplasts regenerating between 39 and 48 hours after isolation, still shows alignment to the direction of the initial unidirectional light stimulus although the stringency of the alignment is diminished (c.f. mean angles of deviation 0-15 hours: 18°; 39-48 hours: 32° – a random distribution would give a figure of 45°). All the protoplasts in this cohort fixed the alignment of their regeneration axes more than 15 hours after removal from the unidirectional light field.

Data from similar experiments where protoplasts were transferred after 15 hours regeneration in unidirectional red light, to darkness are summarised in Table 5B. Here too, axis alignment continues to be influenced by the initial period of incubation in unidirectional light. The axes of protoplasts regenerating 33-57 hours after removal from unidirectional red light show a mean deviation from the light direction of 32°, considerably

**Table 5. Effect on the alignment and polarity of regeneration axes of transfer after 15 hours of regeneration in unidirectional red light to non-directional light conditions***

| Interval (hours after isolation)† | Regeneration (%) | Axis alignment (mean angle of deviation in degrees) | Axis polarity (% axes towards light source) |
|---|---|---|---|
| A. Transfer to omnidirectional white light | | | |
| 0 to 15 | 4 | 18 | 68 |
| 15 to 20 | 12 | 20 | 59 |
| 20 to 25 | 17 | 23 | 63 |
| 25 to 39 | 37 | 29 | 40 |
| 39 to 48 | 14 | 32 | 41 |
| B. Transfer to darkness | | | |
| 0 to 15 | 2 | 18 | 65 |
| 15 to 25 | 15 | 20 | 55 |
| 25 to 39 | 14 | 23 | 51 |
| 39 to 48 | 22 | 27 | 56 |
| 48 to 72 | 38 | 32 | 24 |

*For details of light treatments, see Materials and methods section.
†Data for a time interval were computed by subtracting the distribution at the beginning of the interval from that at the end (see Materials and methods).

**Table 6. The effect of reorientation in or removal from unidirectional light on the polarity of regeneration axes of protoplasts regenerating between 39 hours and 48 hours after isolation**

| Unidirectional light* | Axis polarity (% axes towards light source) | | |
|---|---|---|---|
| | A | B | C |
| Red | 77 | 41 | 46 |
| Blue | 87 | 52 | 61 |
| White | 95 | 68 | 87 |

*For details of light treatments, see materials and methods section.
Column A: protoplasts regenerating undisturbed in unidirectional light.
Column B: protoplasts regenerated in unidirectional light for 15 hours after isolation and then transferred to omnidirectional white light (75 µmol quanta/$m^2$/second).
Column C: protoplasts regenerated in unidirectional light that were reoriented by 90º 15 hours after isolation; polarity is with respect to the second light direction.

below that generated by a random alignment. These protoplasts fixed the alignment of their axes at least 24 hours after removal from directional light.

Transfer from unidirectional red light to either omni-directional white light or to darkness also disrupts the determination of axis polarity. Late-regenerating protoplasts no longer show the polarity bias that is characteristic of regeneration in constant unidirectional red light. Transfer to either omni-directional white light or to darkness leads rapidly to a loss of polarity bias (if polarity is random, 50% of regeneration axes are expected to be oriented towards and 50% away from the direction of the light).

Protoplasts were also regenerated in unidirectional blue or white light for 15 hours and then transferred to omni-directional white light. The detailed results of these experiments are not included but Table 6 summarises relevant data. Essentially similar results to these were obtained when protoplasts were transferred from unidirectional blue or white light to darkness (data not shown). The effect on axis alignment of transfer from either unidirectional blue light or unidirectional white light to either omnidirectional white light or to darkness is less evident. Axis alignment is much less well defined in constant unidirectional light, either blue or white (mean angles of deviation in range 34° to 38°) and transfer to non-directional light conditions results in the mean angle of deviation increasing towards 45°, the value to be expected for a random distribution. Transfer from unidirectional blue or white light does not disrupt axis polarity as rapidly as do similar treatments following exposure to unidirectional red light.

## Programming the determination of axis alignment and polarity

The alignment and polarity of the regeneration axis is affected differently by different light conditions. We propose that the determination of axis alignment and axis polarity is dependent on the establishment of separate gradients that are formed in non-uniform light conditions. Axis alignment is determined before polarity and it is likely that the two processes involve gradients having at least some components that are different.

The patterns of axis alignment and polarity observed in different light conditions can be modelled as follows:

(i) Axes are aligned parallel to the light direction more precisely in unidirectional red light than in unidirectional white or blue light. We therefore propose that the gradient responsible for axis alignment is steeper in red light than in blue or white light. Axis polarity is more sharply defined in white or blue light than in red light, and so we propose that the gradient responsible for polarity is shallower in red light than in white or blue light.

(ii) The alignment of the regeneration axis is first fixed in response to the gradient responsible for axis alignment.

(iii) Outgrowth then occurs at the end of the axis with the higher value of the polarity gradient (it will be assumed throughout that the gradient responsible for polarity is that of an inducer, if however an inhibitor were involved, then outgrowth would be at the end of the axis with the lower value). In constant unidirectional blue or white light, all protoplasts have a sufficiently high polarity gradient to develop towards the light source. A variety of explanations may be advanced to explain the distribution of polarities observed in constant unidirectional red light, where about 70% of protoplasts regenerate towards the light source and about 30% regenerate away. It may be that in unidirectional red light, only 40% of protoplasts establish a polarity gradient, the rest polarizing their axes randomly along the alignment axis. Alternatively, in red light the polarity gradient is not well established such that 30% of protoplasts have a higher value away from, rather than towards the light source. It may, however, be that the interpretation of a shallow gradient is not always precise and there is a 30% probability of a protoplast regenerating at the low end of gradient.

(iv) Reorientation in unidirectional light results in a revision of the two gradients. The model advanced above assumed that the revision of the alignment gradient was rapid and that the alignment of the axis was fixed 8-9 hours before protoplast regeneration. The sharp switch from alignment to the original light direction to alignment to the second light direction is consistent with a rapid establishment of the new gradient following reorientation. It is possible, however, that some of the 8- to 9-

hour period is needed for the axis to be redefined following reorientation and that the fixation of axis alignment occurs somewhat later.

(v) The polarity-determining gradient is established more slowly following reorientation. In red light, where only a shallow gradient is formed, the gradient formed in response to the original light direction, declines sufficiently for the pattern of polarity to begin to be affected in protoplasts that had fixed the alignment of their axes before reorientation. The polarity gradient formed in response to the new light direction is not established sufficiently quickly after reorientation for the normal pattern of polarity along the new axis to be observed during the time span of the experiments reported here. A model for the effect of reorientation in unidirectional red light is depicted graphically in Fig. 5.

(vi) In white light, the polarity gradient is steeper and is established sufficiently rapidly for protoplasts regenerating late after reorientation to show the normal pattern of polarity to the second light direction. The experiments reported here for blue light, suggest that the new polarity gradient may take longer to establish following reorientation than in white light. This would be consistent with the polarity-determining gradient being shallower in blue light than in white light (but steeper than in red light).

(vii) The experiments reported here show that protoplasts that have been regenerated initially in unidirectional red light, retain a memory of light direction for a considerable period following removal from directional light. This observation supports the conclusion that the gradient responsible for the alignment of the regeneration axis is established more steeply in red light. Further it suggests that the rapid revision of the gradient that determines axis alignment, following reorientation in unidirectional light must involve an active process that does not occur in uniform light environments.

(viii) Transfer from unidirectional red light to either omnidirectional white light or to darkness will cause the already weak polarity gradient to decay, leading to the observed randomisation of axes polarity.

(ix) Because the polarity gradient established in blue or white light is steeper than that established in red light, it is to be expected that the bias in polarity will be lost more slowly upon removal from directional blue or white light than from red light. This is indeed observed, with the loss of polarity bias occurring somewhat more slowly when protoplasts are transferred from unidirectional white light than when transferred from unidirectional blue light. This supports the conclusion from reorientation experiments that the steepest polarity-determining gradient is established in unidirectional white light.

## Concluding remarks

The effect of an external light gradient on axis development in regenerating moss protoplasts shows similarities to observations of axis formation in *Fucus* zygotes (Goodner and Quatrano, 1993). Within a time period in which the polar axis is labile, altering the direction of a light gradient can reorient the axis in both systems. Both *Fucus* zygotes and moss protoplasts have a memory of a light gradient, since directional light does not have to be present to maintain the alignment of the axis. Furthermore, the time at which the alignment of the axis is fixed, i.e. cannot be reoriented by changing the direction of the light source, can be identified in both systems.

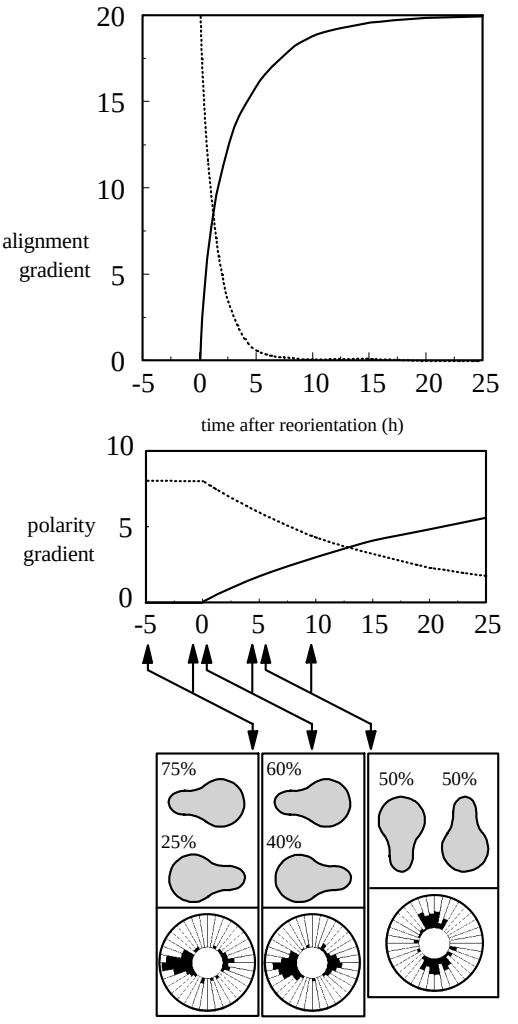


**Fig. 5.** Model for the determination of the alignment and polarity of the regeneration axis in protoplasts regenerating in unidirectional red light, reoriented 15 hours after isolation. The figure models the effect of reorientation with light originally from the left of the figure, and after reorientation, from the top. The upper graph depicts the proposed gradient that determines axis alignment and the lower graph depicts the proposed gradient that determines axis polarity. The broken lines are the gradients along the line of original light direction and the continuous lines the gradients along the line of the new light direction. The absolute values are chosen arbitrarily, and only their relative values are relevant to the model. At the bottom, is a plan of the history of three cohorts of protoplasts regenerating at 4, 9 and 14 hours after reorientation. For each cohort, the upward pointing arrow to the left indicates the time at which axis alignment was fixed, the upward pointing arrow to the right indicates the time at which axis polarity was fixed, and the downward pointing arrow indicated the time at which protoplasts could be seen to have became polar. Axes are aligned along the steepest value of the alignment gradient at the time of axis fixation. Axis polarity is then determined along the aligned axis. When the difference between the values of the polarity gradient at either end of the aligned axis is 8, then 75% of protoplasts regenerate towards the higher end of the gradient and 25% towards the lower. When the difference is less than 4, there is no preferred polarity (50% towards and 50% away from the light direction). Differences above 4 and below 8 result in an intermediate distribution. The circular histograms, taken from Fig. 4, are included for illustration and do not correspond strictly to the cohorts of protoplasts examined.

Although the determination of axis orientation in *Fucus* zygotes and in protoplasts of *Ceratodon purpureus* show similarities, it is clear that the process in the latter is different and probably more complex. In the *Fucus* zygote, the polarity and alignment of the axis are fixed together. Polarised growth always occurs from the shaded side of the zygote and a single process appears to determine both the alignment of the axis of asymmetry and its polarity. In regenerating moss protoplasts however, the development of asymmetry takes place in two steps, with axis alignment being determined before axis polarity. The site of protonemal emergence along the axis is not always set relative to the light gradient, i.e. emergence can be away from, instead of towards the light source. Furthermore, unlike the determination of axis polarity in *Fucus*, reorientation in or removal from a directional light field profoundly disturbs the determination of polarity in regenerating moss protoplasts.

It has recently been demonstrated that in the developing *Fucus* zygote, fluorescent dihydropyridine (FL-DHP), a compound known to label mammalian calcium channels, binds primarily to a membrane component that becomes localized prior to axis fixation (S. L. Shaw and R. S. Quatrano, unpublished data). Localization of this compound is actin-dependent and reorientation of zygotes with respect to light before axis fixation, leads to the relocation of this component. FL-DHP is therefore a marker for axis formation. It would be interesting to determine whether FL-DHP can be used to identify the orientation of the future regeneration axis in moss protoplasts and whether it could be used to predict polarity as well as orientation.

We have shown that it is possible to model the effect of both removal from, and reorientation in, directional light fields, on regenerating *Ceratodon* protoplast, by assuming that two independent gradients determine alignment and polarity. The steepness of these gradients varies differently in different light conditions and the two gradients have different stabilities. Our proposals do not exclude the possibility that the processes of signal transduction that lead to the formation of these two gradients, share common steps and may be initiated by the same photoreceptor. We propose to investigate this possibility further by the isolation of mutants impaired in axis orientation. Mutants unable to align their regeneration axis could be selected by isolating protoplasts regenerating at right angles to unidirectional red light, and mutants impaired in the determination of axis polarity, by isolating protoplasts regenerating away from a blue or white light source.

It is unlikely that other eukaryote systems used for the study of polarity are less complex, rather that the tractability of the protoplast regeneration system to experimental observation has allowed this complexity to be revealed. Furthermore, molecular genetic techniques are already well established for *Physcomitrella patens* (Knight, 1994) and are being established for *Ceratodon purpureus*, and this, together with the accessibility of moss tissue to cell biological investigation (Doonan et al., 1988), suggests that this system will permit a synthesis to be made between the molecular and cell biological events responsible for the determination of cell polarity.

D. J. C. thanks the Leverhulme Trust and the Free University of Berlin for financial support and the University of North Carolina and the Free University of Berlin for allowing the use of their facilities. R. S. Q. acknowledges support from the National Science Foundation (MCB9318757). We thank Jon Hughes, Celia Knight and Tilman Lampeter for their helpful discussion.

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(Accepted 25 September 1995)