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Non-genic inheritance of cellular handedness

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Summary

Ciliates exhibit an asymmetry in arrangement of surface structures around the cell which could be termed handedness. If the usual order of placement of structures defines a 'right-handed' (RH) cell, then a cell with this order reversed would be 'left-handed' (LH). Such LH forms appear to be produced in *Tetrahymena thermophila* through aberrant reorganization of homopolar doublets back to the singlet condition. Four clones of LH forms were selected and subjected to genetic analysis to test whether this drastic phenotypic alteration resulted from a nuclear genetic change. The results of this analysis indicate that the change in handedness is not due to a genetic change in either the micronucleus or macronucleus. The LH form can, under certain circumstances, revert to the RH form, but typically it propagates itself across both vegetative and sexual generations with similar fidelity.

While this analysis does not formally rule out certain possibilities of nuclear genic control involving regulatory elements transmitted through the cytoplasm, when the circumstances of origin and propagation of the LH condition are taken into account direct cortical perpetuation seems far more likely. Here we outline a conceptual framework centred on the idea of longitudinally propagated positional information; the positive evidence supporting this idea as well as further application of the idea itself are presented in the accompanying paper.

Key words: pattern formation, inheritance, cytoplasmic, ciliates, patterning.

Introduction

Cytoplasmic localizations are of great importance in embryonic development. These localizations typically are a consequence of the action of the maternal genome in the oocyte or newly fertilized egg. A classic example of such maternal predetermination is the control of the direction of asymmetry of the body and shell of gastropod molluscs (Sturtevant, 1923; Freeman & Lundelius, 1982). In this case, the effect is exerted by way of a stored cytoplasmic product that functions shortly after fertilization to bring about a reversal in the asymmetry of early cleavage that in turn affects the subsequent macroscopic asymmetry (see Freeman & Lundelius, 1982, and references cited therein). In *Drosophila*, a reversal of polarity of up to one-half of the egg can be brought about by mutants at one of several *bicaudal* loci which involve a maternal predetermination that functions before fertilization (Nüsslein-Volhard, 1977; Mohler & Wieschaus, 1986).

In contrast to the maternal predetermination characteristic of embryonic organization, ciliates have been among the classic exemplars of true cytoplasmic inheritance (reviews: Nanney, 1983; Sapp, 1987). The majority of these cases have turned out to be effects of nucleic acids packaged within symbionts or organelles, or to selfsustaining feedback loops (Nanney, 1983; Preer, 1988). There remains, however, a residuum of cytoplasmically inherited conditions in ciliates that has not yet yielded to the hegemony of nucleic acids. This involves the inheritance of patterns of structural organization. The two classic examples are the inheritance of 180°-rotated (inverted) ciliary rows in *Paramecium* (Beisson & Sonneborn, 1965), which was subsequently observed in *Tetrahymena* (Ng & Frankel, 1977), and the inheritance of the homopolar-doublet condition (Sonneborn, 1963). These examples all involve inherited differences in the number or spatial orientation of structures (or sets of structures) that show normal internal organization.

Recently, investigators in several laboratories have found ciliate forms in which a large part of the cell surface exhibits an arrangement of structures that is a mirror image of the normal, much as in *bicaudal Drosophila*. These include mirror-image *janus* forms in *Tetrahymena* (Jerka-Dziadosz & Frankel, 1979) and mirror-image doublets in *Stylonychia* and its close relatives (Tchang *et al.* 1964). In these forms, only the global arrangement of structures is reversed, while the local architecture within each structure is not reversed (Grimes *et al.* 1980; Jerka-Dziadosz, 1981, 1983; Frankel *et al.* 1984). However, there is no apparent uniformity in the origins of these mirror-image forms: while the *janus* condition in *Tetrahymena* results from the action of recessive mutations at particular gene loci (Frankel & Jenkins, 1979; Frankel & Nelsen, 1986*b*; Frankel *et*

al. 1987), mirror-image doublets in *Stylonychia* are generated by microsurgical operations carried out on normal wild-type cells (Tchang *et al.* 1964; Shi, personal communication).

Still more recently, phenocopies of the *janus* configuration were discovered in *Tetrahymena* cells regulating from a homopolar-doublet to a normal singlet condition (Frankel & Nelsen, 1986*a*). In the course of that investigation, occasional *reverse* singlets were observed (Nelsen & Frankel, 1986), presumably resulting from preservation of the 'wrong' handedness during regulation from a *janus*-like condition to a singlet state. Contrary to early expectations (Frankel, 1984), such 'left-handed' cells could feed and grow, allowing selection and maintenance of clones of these cells. At about the same time, Suhama generated a clone of reverse singlet cells in *Glaucoma* (a close taxonomic relative of *Tetrahymena*) by longitudinal transection of mirror-image doublets (Suhama, 1985). In that case, the 'left-handed' singlet was clearly of non-genic origin, but the origin of the preceding mirror-image doublet clone was unknown (Suhama, 1982).

The capacity to clone and maintain cells with a reversed handedness in arrangement of structures in *Tetrahymena thermophila*, a genetically domesticated ciliate, provided an opportunity for analysis of the inheritance of these differences. This paper is a report of such a genetic analysis. Here we present evidence for the conclusion that the difference in cellular handedness is not due to a difference in nuclear genes. Since the emphasis of this paper is genetic, the reversed phenotype itself will be described schematically without supporting documentation, and only the general concept underlying our developmental model will be presented in the Discussion. The accompanying paper (Nelsen & Frankel, 1989) will provide the details of the phenotypic differences between 'right-handed' and 'left-handed' cells, the consequences of these differences, and a reconstruction of the detailed course of events during changes in cellular handedness.

Materials and methods

Stocks, media, general procedures

Tetrahymena thermophila was used in this study. Wild-type cultures were from the 20th inbred generation, established in 1979 (B2079). Mutant stocks used were IA-104, IA-121, IA-267 and IA-330. IA-104 and IA-121 are homozygous for recessive, temperature-sensitive mutations, *cdaA1* and *cdaC2*, respectively, in which cell division is arrested at restrictive temperatures (Frankel *et al.* 1976). IA-267 and IA-330 both are 'homozygous functional heterokaryon' (Bruns & Brussard, 1974) stocks, homozygous for cycloheximide resistance (dominant) in the micronucleus and cycloheximide sensitivity in the macronucleus [*ChxA2/ChxA2* (cycl.-s)], and of mating types III and V, respectively. In addition, a B* (B-star)-VII stock (Doerder & Berkowitz, 1987), with a defective micronucleus that does not contribute a pronucleus during conjugation, was obtained from Dr F. P. Doerder.

Stocks were routinely maintained axenically at 19 °C in 5 ml tubes containing 1 % proteose peptone plus 0·1 % bacto yeast extract (1 % PPY). Homopolar doublets (see below) were maintained in 2 % proteose peptone plus 0·5 % bacto yeast extract (2 % PPY). A richer medium (PPYGFe, described in Nelsen *et al.* 1981) was used to screen for LH (reverse) cells and to maintain them after selection. Dryl's salt solution, made up as described by Nelsen & DeBault (1978), was used to prepare cells for conjugation.

The standard technique for carrying out conjugation involved washing cells three times and resuspending them overnight in Dryl's salt solution before mixing with appropriate mating types in non-shaking cultures at 30 °C. Cell densities for conjugation were $8–20\times10^4$ cells ml^{-1}. Isolated conjugating pairs were kept at room temperature. These basic procedures were modified for specific purposes, as indicated below.

Assessment of cell-surface geometry was carried out using two silver-staining procedures: Chatton–Lwoff silver impregnation using the procedure of Frankel & Heckmann (1968) as modified by Nelsen & DeBault (1978), and protein–silver (protargol) staining following the general methods of Ng & Nelsen (1977) with the improvement of Aufderheide (1982) except that a thick albumin film was used.

Procedures for selecting LH clones

All LH clones were obtained from homopolar doublets (RH cells fused side by side). Homopolar doublet stocks were constructed using three techniques. The first two have been described previously (Nelsen & Frankel, 1986). In brief, the doublets from which the LH1 clone was obtained were selected from a vegetative culture of stock IA-104 [*cdaA1/cdaA1* (II)] after subjecting it to restrictive conditions (36 °C for 2 h) which arrest cleavage; the doublets that served as the foundation for the LH2 clone were selected from wild-type cells [B2079 IV×VI, locally constructed stocks which are isogenic but of different mating types] fused during conjugation using immobilization antiserum. The doublets used to select the LH3 and LH4 clones were obtained from the homozygous functional heterokaryon stocks IA-267 and IA-330 using a new procedure. Cells of these two stocks were allowed to conjugate for 5 h in Dryl's' salt solution at 30 °C. Conjugation then was blocked by subjecting the culture to 40 °C for 1 h and 39 °C overnight in Dryl's solution. The cells were fed with 2 % PPY the next day and 'stuck' pairs were isolated after 3 to 4 h of growth at room temperature. Many of these yielded doublet cultures. Subcultures of the doublet clones selected were tested for fertility and retention of their heterokaryon character. All doublet clones were originally isolated by visual selection, and subsequently subcloned for weeks to months by selection of doublets every 2 to 3 days. Details of this procedure were reported previously (Nelsen & Frankel, 1986).

To obtain LH singlets, the 'Poisson lottery' techniques of Orias & Bruns (1976) were employed to dispense samples of doublet subclones into microtitre plates in PPYGFe medium containing antibiotics (1·4 g penicillin G potassium and 2·2 g streptomycin sulphate per litre). After growth for 4 to 7 days at room temperature or 3 days at 25–27 °C, plates were screened to select for clones showing slow growth. Slow growth was expected because LH cells commonly have defective oral structures. The slow-growing clones were subcloned and samples stained to check for LH (reverse) geometry. Growth for 3 days at 25–27 °C proved to be optimal for finding these cells. Most of the slow-growing clones were not reversed; however, with experience one learns to recognize LH cells by their swimming behaviours: they often 'hesitate' and/or exhibit a 'twisty' spiralling during forward swimming.

LH clones were maintained by weekly subcloning in an

'Edgegard' (Baker Co., Sanford, Maine, USA) laminar flow hood into tubes containing 5 ml of sterile PPYGFe (without antibiotics) kept at room temperature. For long-term maintenance, stocks were kept in liquid nitrogen using the procedures of Simon & Flacks with minor modifications (1975).

Crosses involving LH clones

Crosses using stocks carrying temperature-sensitive cell-division arrest mutations (IA-104 and IA-121) were carried out using standard procedures (see above). In these LH×RH and control RH×RH crosses, numerous pairs were seen in all cultures one day after mixing. These cultures were maintained at 30°C, and, after three days, nutrients (2% PPY) were added. Subsequently, cultures were shifted to 39°C, and scored for the presence of growing cells 3 days later.

Crosses between LH1 and LH2 stocks were carried out using standard procedures with one exception. To promote mating (costimulation) in the LH cells, a small proportion (1:10) of a third cell culture (B* VII) was added. These are RH cells that lack a functional micronucleus. In these crosses, pairs were isolated, grown in PPYGFe at room temperature and tested for capacity to mate with mating type testers within 15 fissions after conjugation. All 'mature' conjugating lines were presumed to have retained their old macronuclei following an abortive conjugation (Allen & Gibson, 1973).

Crosses between the LH3 and LH4 stocks were carried out by the same general procedures used in the LH1×LH2 crosses. Two subclones of each of the LH parents were prepared for mating and B* costimulators were added to the mating mixture. However, instead of isolating pairs, cycloheximide dissolved in nutrient (2% PPY) medium (final concentration of 30 $\mu g\,ml^{-1}$) was added 32 h after mixing. Surviving LH cells were isolated, grown in PPYGFe and tested as illustrated in Fig. 3 and described in the Results.

Results

Selection of LH clones

Four clones of singlet cells displaying an apparent right–left reversal of global asymmetry were isolated from doublet cultures using a screening protocol which selects for slow growth. The first clone of reverse singlets (designated LH1) was isolated in 1983 from a doublet culture produced through a temporary fission arrest in cells homozygous for the temperature-sensitive cell-division-arrest mutation *cdaA1*. This conditional mutation, under permissive conditions, has no known effect on the arrangement of cell structures. LH1 is obviously vegetative in its origin and retains the mating type II of the parental stock.

The second clone of LH phenotype (LH2) was isolated in 1984 from a doublet clone produced by fusion of conjugating wild-type cells after treatment with homologous immobilization antiserum. The LH2 cells retain the mating type of one of the 'parental' clones, indicating a probable vegetative origin.

The LH3 and LH4 clones were isolated in 1987 from doublet cultures produced by heat treatment of conjugating functional heterokaryon stocks. These two LH clones were, as expected for heat-treated conjugating cells (Scholnick & Bruns, 1982*a*,*b*), vegetative 'progeny' of abortive conjugation, retaining both the drug-sensitivity (cycloheximide-sensitive) and the mating types (III and V, respectively) determined by the macronuclei of the parental cells.

The feature of crucial importance for this investigation is the vegetative origin of all of the LH clones. This vegetative origin can be considered certain for clones LH1, LH3 and LH4, and probable for clone LH2.

The LH phenotype

Some major differences between the typical 'right-handed' (RH) phenotype and the reverse 'left-handed' (LH) phenotype are presented schematically in Fig. 1. The evidence for these and other differences is pre-

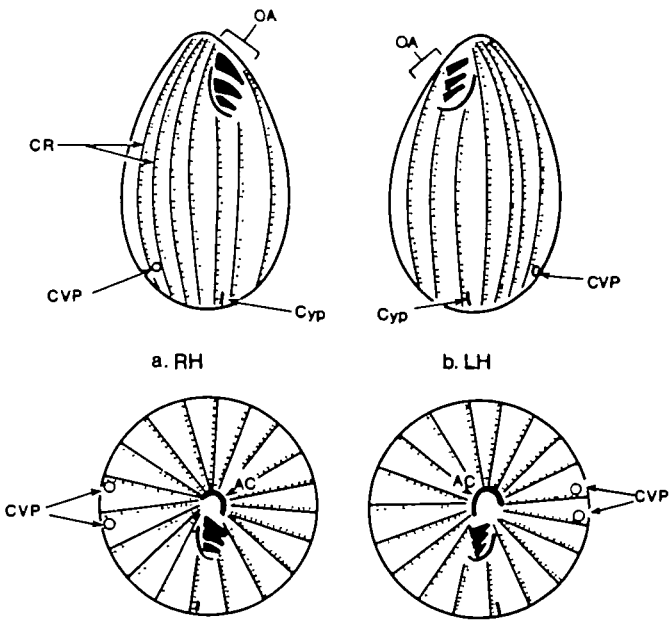


Fig. 1. Diagrams of the cell-surface organization of (a) 'right-handed' (RH) and (b) 'left-handed' (LH) cells of *Tetrahymena thermophila*. Schematic ventral views are shown in the top row, polar projections in the bottom row. In the polar projections, the anterior pole is at the centre, the posterior pole at the margin. The diagrams show the ciliary rows (CR), with longitudinal microtubule bands (lines) always to the cell's right (viewer's left) of the basal bodies (dots). Both RH and LH cells have two postoral ciliary rows, located posterior to the oral apparatus (OA). The cytoproct (Cyp, cell anus) is found along the right postoral ciliary row in RH cells, and along the left postoral row in LH cells, but always to the cell's *left* of the respective postoral ciliary rows. Similarly, the CVPs are in mirror-image positions relative to the OAs in RH and LH cells, but always just to the cell's left of ciliary rows. The apical crown (AC) of basal body doublets, drawn in a highly schematic form in the polar projections, also is in a mirror-image configuration in RH and LH cells. The typical arrangement of structures of the OA is shown for the RH cells, while only one of several arrangements found in LH cells is shown. The individual structural elements within the OA are rotational permutations, not true mirror images, of the corresponding normal structures. (see Nelsen & Frankel, 1989 for a general account; Nelsen *et al.* 1989 for details).

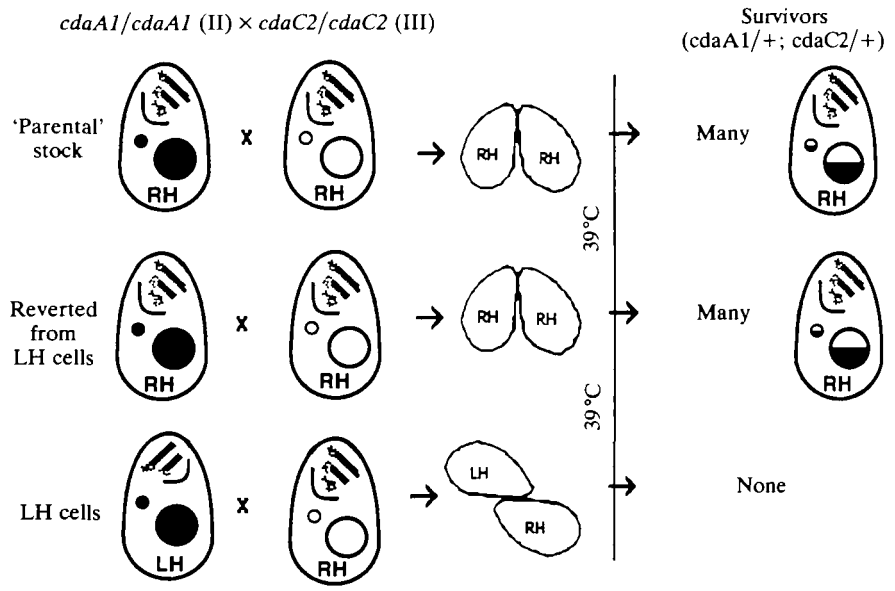


Fig. 2. Crosses testing the capacity of LH and RH cells to produce progeny. Roman numerals indicate mating types. The shading of nuclei in these diagrams indicates the genotype at the *cda* loci, with *cdaA1* nuclei shaded and *cdaC2* nuclei unshaded. Nuclei heterozygous at both loci are half-shaded. Only such double heterozygotes can propagate at 39°C ('survivors' in right column). Some cells from both RH×RH crosses survived the nonpermissive conditions, indicating successful conjugation, while the cells from the LH×RH cross all died, indicating that they did not mate successfully. For further explanation, see the text.

sented in the accompanying paper (Nelsen & Frankel, 1989). The arrangement of structures in LH cells is the reverse of that in RH cells. The positions of the contractile vacuole pore (CVP) and cytoproct (Cyp) and the pattern of basal body couplets at the anterior end of the cell all reflect this reversal. At the local level, however, the polarity within the ciliary rows, positions of microtubule bands, kinetodesmal fibres and cortical mitochondria are the same in LH and RH cells.

The reversal of large-scale asymmetry has serious consequences for development of the OA and hence for cell multiplication (Nelsen & Frankel, 1989). LH cells are therefore at a large selective disadvantage relative to RH cells. Hence, to maintain LH cultures one must periodically subclone the LH cells, since occasional RH 'revertants' will overgrow unselected cultures.

Breeding analysis

LH×RH crosses

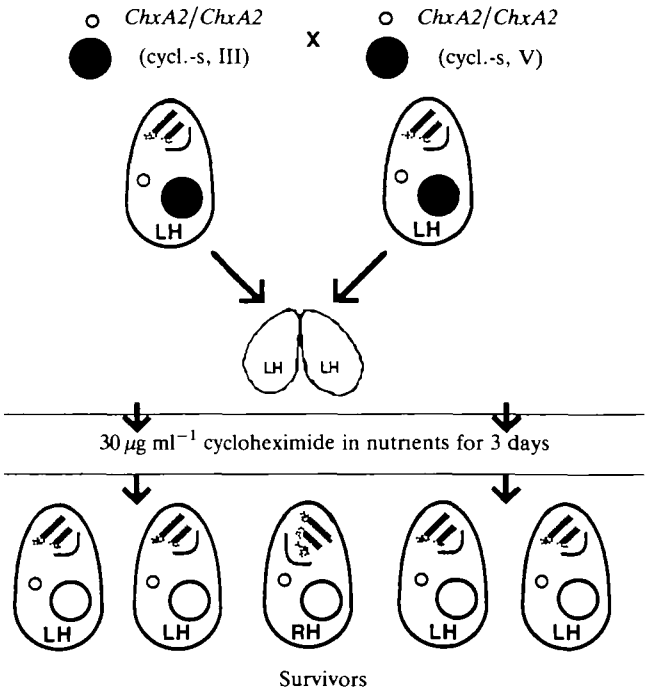
Attempts to cross LH with RH cells proved unsuccessful. A breeding scheme utilizing two recessive temperature-sensitive cell-division-arrest (*cda*) mutations was devised to select for even rare sexual success in an LH×RH cross (Fig. 2). All pairs that had gone through the complete conjugation process would have macronuclei as well as micronuclei heterozygous for both of the recessive mutations (cf. Fig. 4), and hence would continue to divide even at a temperature restrictive for these mutations. Cells that had paired but subsequently aborted the conjugation process and retained the old macronuclei, as well as cells that did not pair, would undergo division arrest under restrictive conditions.

LH cells form heteropolar pairs with RH cells rather than the homopolar pairs normally seen during conjugation. While the mating stimulus sufficed to trigger meiosis and even the development of macronuclear anlagen in some LH×RH pairs (Nelsen & Frankel, 1989), putative exconjugants did not divide under restrictive conditions, indicating a prior failure to produce genuine progeny. Many RH 'parental' cells from which the LH1 clone was derived, and RH 'revertants' from the LH clones, survived the restrictive conditions following conjugation with RH cells, and hence were successful in producing progeny.

Successful mating of RH×RH pairs, while LH×RH pairs of the same genetic stock failed, suggests that the anterior–posterior mismatch of the bonding surface of the heteropolar LH×RH pairs might account for failure to produce progeny (Nelsen & Frankel, 1989). This implies that an LH×LH cross might be successful.

LH×LH crosses

An LH×LH cross is sufficient to test the hypothesis of a nuclear genic origin for the LH condition. The reason for this lies in the vegetative origin of the LH clones, described above. Since it has been demonstrated that when the micronucleus and macronucleus have different genotypes the micronucleus has no effect on the cellular phenotype (*Tetrahymena*: Nanney & Dubert, 1960; Bruns & Brussard, 1974; Mayo & Orias, 1985; *Paramecium*: Sonneborn 1947 p. 270; Tam & Ng, 1987), a mutation present in the *mic*ronucleus alone would not be expressed so long as the macronucleus retained the original wild-type genes specifying the RH condition. It therefore follows that if the origin of the LH condition is based on a nuclear mutation, it must be in the *mac*ronucleus. A dominant macronuclear mutation might come to expression quickly, while a recessive macronuclear mutation could only be expressed after phenotypic assortment (Nanney, 1964; Orias & Flacks, 1975; Doerder *et al.* 1977). The final possibility, of simultaneous independent mutations in *both* the micronucleus and macronucleus, is extraordinarily unlikely on probabilistic grounds. It is important to note that there is excellent genetic evidence that macronuclear



Retest cycl.	Maturity ≈10 fissions	Mating type ≈80 fissions	Handedness, % LH ≈100 fissions
All resistant	All immature	9 – IV 5 – II 3 – VI 1 – III	12 ≥ 90 2 – 80–89 2 – 70–79 1 – 60–69 1 – 50–59

Fig. 3. LH×LH crosses to test the possible mutational origin of the LH phenotype. Homozygous functional heterokaryons, *ChxA2/ChxA2* (cycl.-sens) [cycloheximide resistant micronucleus (sensitive macronucleus)] of two mating types (indicated by Roman numerals) were utilized (the B* cells used as costimulators are not shown). The shading of nuclei indicates the genotype at the *ChxA2* locus, with the genotype conferring sensitivity shaded and genotype conferring resistance unshaded. Only cells that undergo complete conjugation (Fig. 4), forming new macronuclei from micronuclear sources, can survive the cycloheximide poisoning. The survivors included many LH cells, which after subcloning maintained their LH character. This result refutes the hypothesis that the LH phenotype is based on a mutation that originated and was maintained in the macronucleus. For further explanation, see the text.

genetic changes in T. thermophila do not secondarily affect micronuclei during vegetative growth (Nanney & Dubert, 1960; Bruns, 1986), although in certain unusual cases they may affect new macronuclei during conjugation (Doerder & Berkowitz, 1987).

The logic of the test for a nuclear genic origin of the LH condition is shown in Fig. 3. Since any putative LH mutation must be in the macronucleus but not the micronucleus, all true progeny of this cross, which have formed new macronuclei from a zygotic micronucleus (cf. Fig. 4), should have the wild-type (RH/RH) genotype, and hence should revert to the RH form.

Preliminary tests with two LH clones (LH1 and LH2) of different mating types yielded few pairs, which tended to fall apart on attempted isolation. These failures presumably resulted from difficulty in pairing due to a short preoral suture in LH cells (Nelsen & Frankel, 1989). To help overcome this problem, RH cells of a third mating type were added as costimulators (Bruns & Palestine, 1975; Suganuma *et al.* 1984) to promote the development of mating surfaces. This third mating type was a B* (B-star) (VII) clone that lacked a functional micronucleus, and would ordinarily yield no progeny except through an alternative form of conjugation known as 'genomic exclusion' (Allen, 1967), which involves two successive rounds of mating before a new macronucleus can be produced. In these LH×LH crosses, no genuine progeny were obtained following pair isolations, but assessment of protargol-stained mass cultures showed that some LH–LH pairs were proceeding through the nuclear stages diagnostic of normal conjugation. However, by the time this assessment was made the LH2 clone was showing indications of general decline (probably independent of the LH condition), so new LH clones were selected.

The definitive test then was carried out with the newly constructed LH3 and LH4 clones. These clones were 'homozygous functional heterokaryons' (Bruns & Brussard, 1974), which were homozygous for a dominant allele (*ChxA2*) conferring cycloheximide resistance in the micronucleus and the recessive allele conferring cycloheximide sensitivity in the macronucleus (Fig. 3). In this scheme, only cells that develop new macronuclei can survive the cycloheximide poisoning (Bruns & Brussard, 1974). Since the B* costimulators are cycloheximide-sensitive, they could survive cycloheximide poisoning only after conjugating with RH 'revertants' (recall that RH×LH crosses do not yield progeny); they would ordinarily have to do so twice in succession to produce cells with new macronuclei [except possibly in rare cases of 'short-circuit genomic exclusion' (Bruns *et al.* 1975)]. Thus their contribution to the population of survivors after cycloheximide poisoning probably is negligible.

Four crosses between the LH3 and LH4 clones were carried out. In each of these crosses, both LH and RH survivors were observed after 3 days of growth. The surviving RH cells may have resulted from the mating of 'reverted' RH cells of the starting cultures with each other or possibly from rare cases of matings with the defective costimulators. Nine surviving putative LH cells were isolated for each of the four crosses. Of these 36 cells, four died, and four clones appeared (by inspection) to be RH in character and were discarded. The 28 remaining clones were immature, cycloheximide resistant and LH in character. Ten of these clones died during the subsequent subcloning, while 18 were carried through all of the tests indicated in Fig. 3.

The demonstration of cycloheximide resistance, immaturity (initial inability to mate) and new mating types upon subsequent maturity indicates that new

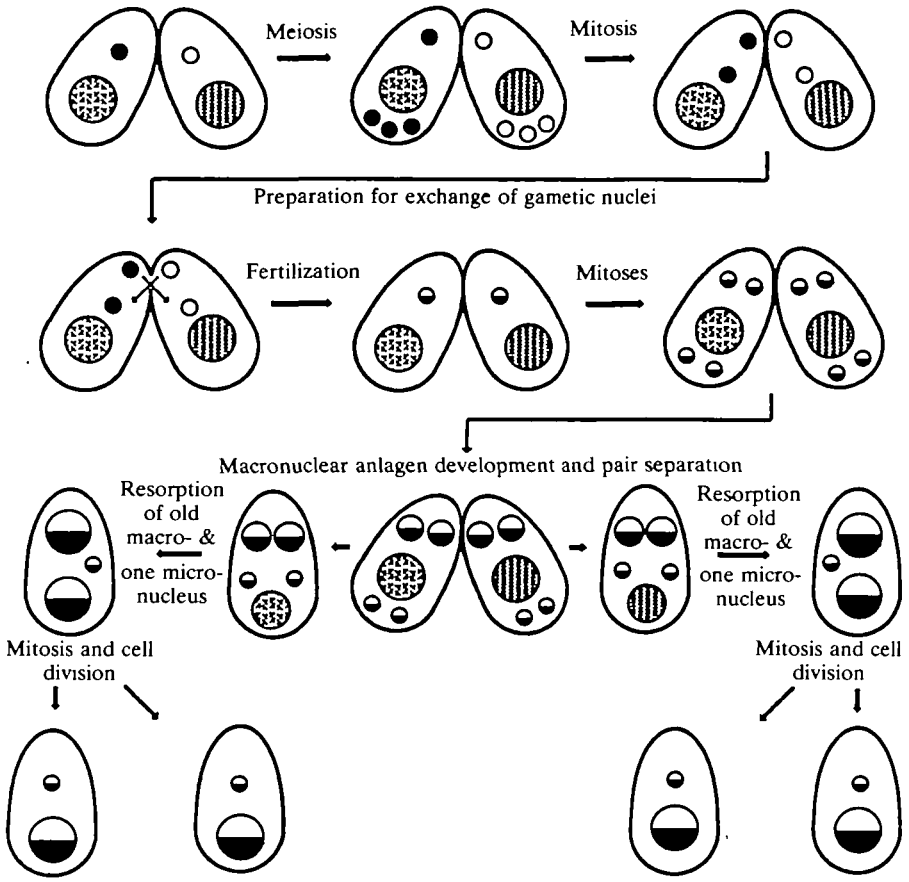


Fig. 4. A schematic diagram of conjugation between *Tetrahymena* cells with differing micronuclear and macronuclear genotypes. Here, *all four* of the nuclei involved are of differing genotypes. The micronuclei differ in alleles at one or more loci unrelated to the LH phenotype, while the macronuclei are assumed to have acquired mutations that specify the LH phenotype. The micronuclei of the two conjugating cells enter meiosis synchronously. Three of the four products of meiosis are resorbed, while the remaining one divides mitotically to produce two gametic nuclei. These are exchanged reciprocally. Each gametic nucleus from one cell fuses with a gametic nucleus from the other cell, to form zygotic nuclei that are genically identical in the two cells. The zygotic nuclei then undergo two postzygotic divisions. Of the resulting four nuclei in each conjugant, two form macronuclear anlagen that develop into new macronuclei, one forms the new micronucleus, and one is resorbed. The old macronuclei also are resorbed. The two new macronuclei of each exconjugant are segregated to two daughter cells. The new micronuclei and macronuclei are heterozygous for the allelic differences between the micronuclei of the preceding sexual generation; however, they do not carry any of the macronuclear mutations from the previous sexual generation, including the putative mutations presumed to give rise to the LH phenotype. For a detailed description of conjugation in *Tetrahymena*, see Orias (1986).

macronuclei *were* formed in these cells. These nuclei were formed either by normal exchange of gametic nuclei between partners or by fusion of gametic nuclei without exchange (cytogamy) (Orias *et al.* 1979). In either case, all macronuclear mutations should have been eliminated, thereby removing any nuclear genetic basis for the LH form. Yet LH forms continued to predominate at the time of quantitative assessment 100 generations after conjugation in all of the 18 clones (Fig. 3) and persisted at 140 fissions when most of the clones were discarded and samples of three clones were selected for cryopreservation. The LH condition was also maintained in these three 'stock' clones for 250 to 300 generations postconjugation. These results indicate that the LH condition is not a simple consequence of a nuclear mutation.

Discussion

A non-genic basis for cellular handedness

In this study, we have demonstrated that the propagated difference between two mirror-image arrangements of cell structures on the surface of *Tetrahymena thermophila*, the 'right-handed' and 'left-handed' arrangements, are not based on differences in nuclear genes. This demonstration required a somewhat unconventional procedure. A standard cross-breeding analysis in ciliates is based on three aspects of conjugation: temporary union of conjugating cells, reciprocal fertilization of gametic nuclei derived from micronuclei, and development of new macronuclei from mitotic products of the zygotic (micro)nuclei (Fig. 4). Thus the standard way to initiate a genetic analysis is to cross cells of

different micronuclear genotypes, as shown in Fig. 4. A cross-breeding analysis of this type was not possible in our analysis of the difference between RH and LH clones, in part because while RH and LH cells could conjugate they did not yield any viable progeny. We were nonetheless able to carry out a genetic analysis of the LH phenotype by relying on two other features of ciliate genetics: the control of the cellular phenotype by the macronucleus and the loss of old macronuclei during conjugation. Since all of our LH clones originally arose vegetatively, i.e. *without* the formation of new macronuclei from micronuclei, we know that if the LH condition had arisen by mutation, that mutation would have originated in the macronucleus. This is illustrated schematically in Fig. 4 by the hatching of the macronuclei. Since macronuclei are lost during conjugation (Fig. 4), if the LH phenotype depended on a (macro)nuclear mutation, that phenotype should have been lost in *any* cross involving LH cells, including an LH×LH cross. It was not lost, and therefore it could not have arisen from a nuclear mutation.

There is one apparent and one real ambiguity in this demonstration. The apparent ambiguity centres around the instability of the LH phenotype which is evident both before and after the critical LH×LH cross. RH 'revertants' appear sufficiently often that LH clones can be maintained only with frequent selection of LH cells. Does this phenotypic instability have a genic basis? In the case of the *PEP4* gene in *Saccharomyces cerevisiae*, a new (hydrolase-minus) phenotype of recessive *pep4* haploid yeasts derived from *PEP4/pep4* diploids comes to expression after a phenomic lag of 80 generations or more, with sectoring indicative of phenotypic instability (Zubenko *et al.* 1982). However, the LH condition in *Tetrahymena* persists for a longer time after the critical cross – at least 300 generations – than was demonstrated for the hydrolase-plus phenotype in *pep4* yeast segregants. Even more important, unlike the hydrolase-plus phenotype of yeast carrying the wild-type *PEP4* allele in yeast, the LH phenotype of *Tetrahymena never* is truly stable, either before or after the cross. The precise reversion rate per generation is not readily computable from the data in Fig. 3 (the percentage of LH cells was assessed in tube cultures started by 6 cells each), but appears roughly comparable to that of vegetative LH clones. Thus, unlike the yeast example, there is no indication of a major phenotypic difference before and after the cross, and hence no support for the hypothesis of a corresponding nuclear genic difference.

The second and genuine ambiguity concerns the possibility of a *genetic* rather than a phenotypic feedback system: Specifically, while ciliate micronuclei and macronuclei are sealed off from each other during vegetative growth, they are not during macronuclear development. There is a long history of studies in *Paramecium* which demonstrate that pre-existing macronuclei can influence the characteristics of new macronuclei that are formed during conjugation (Sonneborn, 1977); recent evidence shows that this influence affects the genetic structure of the developing macronucleus (Epstein & Forney, 1984; Harumoto, 1986). A similar genetic feedback has recently been discovered in *Tetrahymena* (Doerder & Berkowitz, 1987), although it is not effective in the genetic situation comparable to that of the cross shown in Fig. 3. One could postulate that the LH macronuclear genotype specifies the LH phenotype and also specifies a cytoplasmic state that causes wild-type micronuclei to lose RH-determining base sequences during their development into macronuclei. However, the LH phenotype differs in its persistent vegetative instability from the cases of genetic feedback, in which phenotypic stability is the rule after the macronucleus has become fully differentiated (Sonneborn, 1977).

While we cannot conclusively rule out the possibility that such a genetic feedback system may control the maintenance of the LH condition, both the circumstances of origin and conditions of maintenance of LH clones argue against this alternative. All LH cells were derived vegetatively from RH cells through regulation of homopolar doublets. Furthermore, LH cells probably regulate back to the RH phenotype *via* a homopolar-doublet pathway: the frequency of such reverse-regulation is greatly increased under conditions that promote doublet formation through blockage of cell division (Nelsen & Frankel, 1989). The close connection between the degree of vegetative instability and the pre-existing cellular organization is not characteristic of phenotypes controlled by genes; it suggests a different conceptual model, whose essence will be presented here and whose details will be elaborated in the accompanying paper.

The clonal cylinder and the propagation of large-scale asymmetry

As first pointed out by Tartar (1962), a ciliate clone can be thought of as a cylinder that is growing longitudinally and periodically is segmenting (dividing) transversely (Fig. 5). This geometry lends itself to the inheritance of any cellular feature that is capable of being propagated longitudinally. The best known example, the inheritance of the spatial orientation of ciliary rows (Beisson & Sonneborn, 1965; Ng & Frankel, 1977), can be thought of as a selfperpetuating supramolecular scaffold. The imagery of a selfperpetuating scaffold does not, however, provide a clear explanation for why a structure such as the newly formed oral apparatus, which develops at a considerable distance from any pre-existing structure of the same kind (Fig. 5), should normally be formed along the same cell longitude as the pre-existing structure. Ciliates appear capable of perpetuating not only microscopically visible structural ensembles but also the as-yet-invisible instructions that determine where visible structures such as the oral apparatus and the contractile vacuoles may be formed.

These invisible instructions can be represented as a grid of positional latitudes and longitudes (Fig. 6). The values of the latitudes (letters in Fig. 6) must be respecified in every division (segmentation) cycle, but the longitudes (numbers in Fig. 6) could in principle be perpetuated indefinitely. Further, a single complete set of longitudes can be wound only in two ways around the

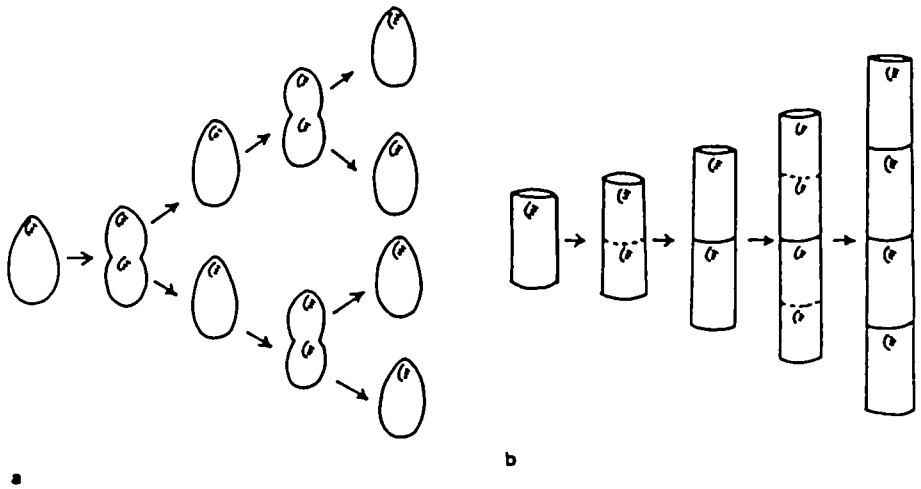


Fig. 5. A schematic of clonal growth in ciliates. (a) Two division cycles in *Tetrahymena*. (b) The same visualized as a longitudinally growing cylinder that undergoes periodic segmentation. Oral structures are shown. From Frankel & Nelsen (1981).

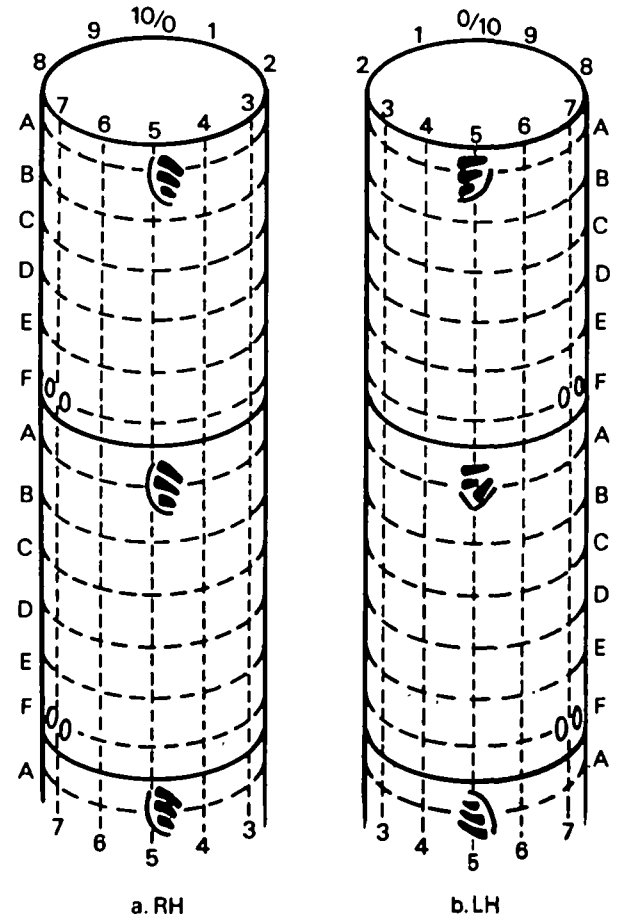


Fig. 6. Orthogonal coordinates superimposed on the clonal cylinder for (a) 'right-handed' (RH) and (b) 'left-handed' (LH) cells. Solid lines indicate cell boundaries, while dashed lines indicate hypothetical latitudes (letters) and longitudes (numbers). Oral apparatuses are shown at the coordinate 'A5', contractile vacuole pores at 'F7'. The oral apparatuses of RH cells are shown in their typical, invariant form, while those of LH cells are drawn to emphasize the large variations in internal organization that are encountered (Nelsen & Frankel, 1989). For further explanation, see the text.

cell, as shown in Fig. 6a and b, respectively. If longitudes were propagated as the cylinder grows, there is no way in which either type of winding could be converted to the other without cutting into the circle and substituting subsets of longitudes.

If we imagine that the different numbers represent positional values that are 'interpreted' to promote formation of different structures (such as the oral apparatus at 5, the CVP-set at 7), the cylindrical topology of ciliate growth would allow for a propagation of these positional values. The direction of winding of the ensemble of positional values would then be the basis for the difference between the RH and LH configuration. This direction would control the arrangement of surface structures and influence the internal organization of complex structures such as the oral apparatus (Nelsen *et al.* 1989). The instability of the LH configuration is a consequence of the topological transitions that are already known to occur in regulating doublets (Frankel & Nelsen, 1986*a*, 1987; Nelsen & Frankel, 1986), amplified by the tremendous growth advantage of RH cells.

The concept of propagated intracellular positional values is based on a transposition of the idea of positional information (Wolpert, 1969, 1971) and its corollary, nonequivalence (Lewis & Wolpert, 1976), to the intracellular realm. It also is specifically related to the 'polar coordinate' model of French *et al.* (1976). Thus, we conceptualize the transitions between RH and LH forms in terms of reverse intercalation within the contracting perimeter of regulating doublets (Nelsen & Frankel, 1989), an intracellular and morphallactic application of the shortest-distance intercalation rule as initially formulated in the polar coordinate model of French *et al.* (1976). This permits us to explain the genically and non-genically inherited differences in large-scale asymmetry within a single model: the non-genically inherited cases involve reverse intercalation provoked by abnormal distributions or confrontations of positional values, the genic cases arise from reverse intercalation provoked by an incapacity of the genetic system to maintain certain subsets of these values (Frankel & Nelsen, 1986*b*). The difference is not in the underlying mechanisms but in the proximate causes for

bringing the mechanisms into play.

This concept can be extended to the speculative idea that the underlying substratum of positional information might be similar in ciliates and eggs (Frankel, 1989, chapter 11). However, even if this is true, there is one essential difference: while the geometry of ciliate growth allows a positional system to extend across division products and thereby perpetuate itself, eggs are formed anew in each sexual generation, with no direct topological continuity to the organization of the mother. For this reason, differences of large-scale asymmetry are directly inheritable in ciliates, while in multicellular organisms they must be rebuilt in every generation, with the choice between alternatives depending on information supplied by the maternal genome. The importance of the ciliate example is that it demonstrates that a system of positional information has the potential for direct inheritance; the usual absence of such inheritance in animals might then be an accidental consequence of the newly evolved geometry of sexual reproduction. In the rare cases of direct asexual propagation, as in the fission of flatworms, the direct perpetuation of positional values might well be retained (Sonneborn, 1930).

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