MODIFIERS OF THE *cot* GENE IN NEUROSPORA: THE GULLIVER MUTANTS[1]

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MORPHOLOGICAL mutants were originally studied in Neurospora on account of their value as genetic markers. Recently, their potential usefulness for the analysis of differentiation has received some attention (for example MURRAY and SRB 1962; SUSSMAN, LOWRY, and DURKEE 1964; BACHMANN 1964; BRODY and TATUM 1966). This is a promising development because a well characterized group of morphological mutants may become instrumental in the elucidation of the circuits involved in the programming of morphogenesis, just as the study of regulatory mutants led to the elucidation of the circuits involved in the control of metabolism (JACOB and MONOD 1961).

This paper deals with a group of morphological mutants which might be useful for the approach outlined above. These mutants act as modifiers of the temperature sensitive colonial gene *cot*, giving rise to giant ("gulliver") colonies.

MATERIALS AND METHODS

Strains: Standard markers were obtained from the Fungal Genetics Stock Center, and combined with the *cot* gene by crossing. *cot* (isolation No. C102t) determines colonial growth at temperatures above 32°C, but unrestricted, wild-type growth at lower temperatures (MITCHELL and MITCHELL 1954). Its wild-type allele, on the other hand, allows unrestricted growth regardless of the temperature. Gulliver *(gul)* mutants were usually isolated by ultraviolet (UV) irradiation of microconidial *(pe fl) cot* strains. The UV doses applied allowed survival of 0.2 to 13% of the population.

Media and incubation temperatures: Fries minimal medium (BEADLE and TATUM 1945) was used for plating at 32 to 34°C. Occasionally, a mixture of 0.1% sorbose, 0.05% glucose and 0.05% fructose was substituted for sucrose in order to obtain colonial growth of *cot*+ strains. The medium of WESTERGAARD and MITCHELL (1947), with 1% sucrose, was used for crosses at 20 to 25°C. The medium of VOGEL (1956) was used for stock cultures at 20°C. Media were supplemented as required.

Heterocaryons: The input strains, carrying different auxotrophic markers, were inoculated jointly on minimal medium plates. Single inoculations served as controls.

Crosses: In all *gul*+ × *gul* crosses, *gul*+ was the protoperithecial parent. Ascopores were collected and treated for 5 min with a solution of sodium hypochlorite. The ascospores were washed in water by centrifugation and resuspended in semisolid agar (0.2%) for counting, activating by heat-shock, diluting and plating. Germination was scored using a dissection micro-

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scope, after 24 hours incubation. Segregation was scored after three or more days. Segregant colonies were isolated under the dissection microscope, taking special care to insure, as far as possible, that each colony originated from a single ascospore.

RESULTS

Growth habit: *gul*$^+$; *cot* colonies reach the stationary phase within 12 hours of germination, while *gul*; *cot* colonies take about five times longer to reach the same stage (Figure 1). (Unless otherwise specified, all strains carried the gene *cot*; thus the symbol *cot* will usually be omitted in designating genotypes.) A further difference between *gul*$^+$ and *gul* relates to the rate of growth prior to the stationary phase, as can be also appreciated in Figure 1. This latter difference may, however, be merely a reflection of the former, since the very early onset of the stationary phase in *gul*$^+$ causes it to merge with the lag phase.

The growth pattern of mutants representing the various *gul* loci (see below), investigated with conidial cultures, is essentially as described above, except in the case of *gul-5*. This mutant is characterized by a delay in the expression of the gulliver character: it starts growing like a typical *gul*$^+$ strain reaching an early stationary phase before producing a burst of gulliver-like growth. No systematic observations were made of *gul* mutants in the absence of *cot*.

Mapping and germination: Out of a sample of 33 independently induced *gul*

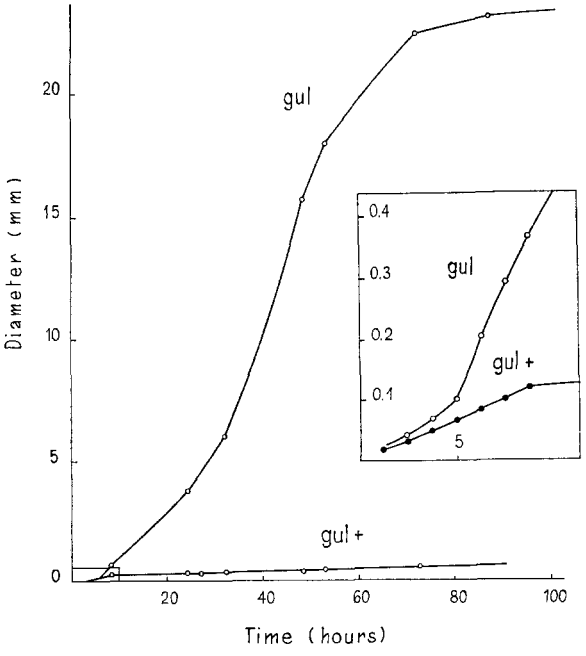

FIGURE 1.—Growth of *gul-1,cot* and *gul-1*$^+$*,cot*, at 33 ± 0.5°C, from ascospores. Time zero is the end of activation. Diameter was measured in the direction of the long axis of the ascospore, and the average of 35 readings was recorded for each time. The ascospores were collected from the cross *gul-1*$^+$*,cot* × *gul-1,cot*, and classified as *gul* or *gul*$^+$ by their ultimate pattern of growth. The early readings are shown in more detail in the inset.

mutants, 25 were located on linkage group V, some 13 units from *inos*. This distance was calculated plating random ascospores from *gul*$^+$ *inos*$^+$ × *gul inos* crosses and scoring gulliver colonies on minimal (331) and inositol (2479) media. The *inos*-linked gullivers appear to belong to a single locus (*gul-1*, formerly called *G*; REISSIG 1958) as judged from the fact that among a dozen different crosses between such gullivers, the yield of *gul*$^+$ recombinants never surpassed 0.1% (MOLINARI, unpublished).

A second locus, *gul-2* (represented by one mutant) is neither linked to *inos* nor to *gul-3*, *gul-4*, *gul-5*, or *gul-6*. *gul-2* has not yet been mapped. All 26 *gul-1* or *gul-2* alleles studied yield regular 1:1 segregations (4:4 in asci) when crossed to *gul*$^+$ strains. On the other hand, the remaining seven gulliver mutants studied yield over 99% *gul*$^+$ colonies in similar crosses. The reason for this anomaly becomes apparent when the germination pattern is examined. Crosses involving nonsegregating gullivers (NS *gul*) are characterized by germination frequencies just about one-half the usual frequencies (Table 1), and asci from such crosses contain at least two pairs of nongerminating (though dark) ascospores. The inference is that NS *gul* alleles do not segregate because they are lethal in the ascospores. Confirmatory evidence will be presented in the section on exceptional gulliver progeny.

No improvement in germination nor in the segregation of gullivers was observed when ascospores from *gul*$^+$ × NS *gul* were activated by furfuraldehyde treatment (one hour in 10^{-3} M) instead of heat shock.

The seven nonsegregating *gul* mutants were mapped on the basis of their lethal phenotype. They define four loci, named *gul-3* to *gul-6*. *gul-3* (represented by one allele) lies on the right arm of linkage group IV. On the basis of the data presented in Table 2 and the general map of this region (MALING 1959; PERKINS, GLASSEY and BLOOM 1962), it can be located half way between *pyr-2* and *cot*.

TABLE 1

Frequency of germination in crosses involving nonsegregating gullivers

	gul$^+$ × NS *gul*		*gul*$^+$ × *gul*$^+$*	
Experiment	Ascospores counted	Percent germination	Ascospores counted	Percent germination
1	180	22	97	67
2	212	24	222	79
3	210	34	...	..
4	238	37	...	..
5	308	37	...	..
3–5	...	..	192	91
6	1096	48	675	95
7	132	51	128	96
8	9298	49	986	96
Weighted average	...	48	...	92

* The *gul*$^+$ × *gul*$^+$ control crosses were run in parallel with the test series. One of the control parents was the same used in the test series. The other control parent was the same strain from which the corresponding nonsegregating *gul* derived by mutation.

TABLE 2

Mapping of gulliver loci on the basis of the lethality of nonsegregating gul *alleles*

Crosses*	Phenotype of random ascospores†		Distance from *gul* to marker (map units)‡	Linkage groups
	Parental combinations	Recombinants		
gul-3+ pt+ cot × *gul-3 pt cot*	4026	552	24	IV
gul-3+ cot+ × *gul-3 cot*	2385	272	10	IV
gul-3+ pyr-2 cot × *gul-3 pyr-2+ cot*	232	21	7	IV
gul-4+ nic-3; cot × *gul-4 nic-3+; cot*	4945	1003	17	VII
gul-5+ tryp-2; cot × *gul-5 tryp-2+; cot*	6872	765	10	VI

* Markers used: *cot:* colonial temperature sensitive, C102t, IVR; *pt:* phenylalanine-tyrosine, S4342, IVR; *pyr-2:* pyrimidine-2, 38502, IVR; *nic-3:* nicotinic-3, Y31881, VIIL; *tryp-2:* tryptophan-2, 75001, VIR.
† Auxotrophs were scored comparing colony counts on supplemented and unsupplemented plates; since nonsegregating *gul* alleles are ascospore lethals, only the segregation of *gul+* need be considered to calculate parental and recombinant types.
‡ Distances were corrected for the ratio of marker alleles segregating in control *gul+* × *gul+* crosses.

gul-4 (four known alleles) maps 17 units from *nic-3*, on linkage group VII; *gul-5* (one allele) lies 10 units from *tryp-2*, on linkage group VI (Table 2). The remaining nonsegregating *gul* mutant is not linked to either *inos* (linkage group V), *cot* (IV), *nic-3* (VII), *gul-2* or *gul-5*, and therefore defines a new locus (*gul-6*).

Forward mutation: The frequency of occurrence of *gul* mutants is high. This can be appreciated from Table 3, where rates of forward mutation at *gul* and *pyr-3N* are compared by scoring for the two characters in the same microconidial population. The high sensitivity of the method for scoring *pyr-3N* mutants (REISSIG, ISSALY, NAZARIO and JOBBAGY 1965) argues against the possibility that this difference be caused by difficulties in detection. Further support for the notion that (some) gulliver loci are highly mutable, derives from consideration of the different loci involved. Out of a sample of 36 UV-induced gullivers, 25 belong to the *gul-1* locus, while 11 map elsewhere. Therefore, *gul-1* is the sig-

TABLE 3

Frequency of gulliver mutants, compared with pyr-3N *mutants*

Mutagen	Lethal hits per microconidium	Number of gullivers counted	Forward mutants × 10^4			
			Gulliver		*pyr-3N**	
			Per survivor	Per lethal hit	Per survivor	Per lethal hit
None	0	13†	0.4†	. .	0.007	. . .
Ultraviolet light	1.3	9	5	4	0.24	0.18
	2.5	15	29	12	0.64	0.26
Ethyl methane sulfonate	1.0	37	6	6	0.24	0.24
	2.5	49	38	15	0.60	0.24
Nitrous acid	1.8	13	11	6	0.14	0.08
	3.3	17	69	21	0.30	0.09
Averages	. .	. .	. .	11	. . .	0.18

* For methods see REISSIG (1963).
† Data from 15 different cultures. Calculation from the number of cultures with no mutants (LURIA and DELBRUCK 1943) yields similar results.

nificantly mutable locus, not only in relation to *pyr-3N* (42-fold more mutable), but also to the other *gul* loci (11-fold more mutable, considering that there are five *gul* loci besides *gul-1*).

Pleiotropy: Besides affecting colony size, mutation from *gul*$^+$ to *gul* has been accompanied by the following phenotypic effects: (a) Ascospore lethality, as described under mapping. (b) Loss of female fertility and of the ability to make heterocaryons, in all mutants at *gul-3*, *gul-4* and *gul-6*, but not at other loci. (c) Four out of a sample of 54 gullivers were colonial at 25°C as well as at 33°C, and a few more showed less distinct tendencies towards colonial growth at the low temperature. Conidiation was reduced in these temperature-insensitive colonials. (These observations were made on strains carrying *cot*. The temperature-insensitive gullivers were not studied further.)

Exceptional gulliver progeny in crosses of nonsegregating gul × gul$^+$: This section refers specifically to results obtained with *gul-3*, *gul-4* and *gul-6* mutants. Results with *gul-5* are at variance and will be described elsewhere. It should be recalled that female sterility prevents crosses of NS *gul* × NS *gul*. Crosses of *gul*$^+$ × NS *gul* will now be considered. The virtual absence of gulliver progeny in such crosses is a consequence of ascospore lethality (c.f. section on mapping). However, a fraction of a percent of gullivers (exceptional progeny gullivers or EPG) do appear in these progenies. This implies that ascospore lethality is not complete. What then might be the cause of this infrequent escape from lethality? The hypothesis was tested that this escape is due to pseudowild type (PWT) formation, *gul*$^+$ being dominant over *gul* with respect to lethality. (Pseudowild-type progeny supposedly arise as heterozygous disomics that break down to form heterocaryons.)

This hypothesis would predict in the first place that EPG would be heterocaryotic for *gul* and *gul*$^+$ nuclei. Synthetic heterocaryons between such nuclei are characterized by displaying *gul*$^+$ morphology in the first 1 to 2 days, followed by a burst of typical gulliver growth. Exceptional progeny gullivers are likewise delayed. Fifty-eight EPG colonies were isolated in order to obtain more direct proof of their heterocaryotic nature. Conidia from each of the resulting cultures were plated, yielding a mixture of *gul* and *gul*$^+$ colonies in the case of 23 EPG cultures, but only *gul* colonies in the remainder. The failure to detect *gul*$^+$ in the latter was not unexpected, because previous experience with synthetic heterocaryons had shown that *gul* nuclei have a selective advantage over *gul*$^+$ under those conditions.

A further prediction implied in the PWT hypothesis is that if the parental cultures were differentially marked, markers located in the same chromosome as the *gul* gene should be heterocaryotic in EPG colonies, while markers on other chromosomes should not. The results bear out these expectations (Table 4), since out of 13 EPG colonies derived from *gul-4*, all markers in the same chromosome as *gul-4* segregated out in the progeny. Of these, in ten instances the parental combinations were preserved, while in three the *nic-3* and the *gul-4* alleles were switched as expected if reciprocal recombination had occurred (PITTENGER and COYLE 1963). In the same crosses, only one instance of mosaicism for a marker

TABLE 4

Marker mosaicism in exceptional-progeny gulliver colonies, as proof for their origin as pseudowild types

Gulliver parent		Number of EPG colonies bearing same or different markers in their *gul* and *gul*+ components			
		Linkage group VII markers		Markers in linkage groups I, II or V	
Locus	Linkage group	Same	Different	Same	Different
gul-4	VII	0	13	12	1*
gul-5	VI	7	2*	9	0

* Different for only some of the markers.
This table pools results obtained with the following crosses:
gul-4+ *nt*+ *nic-3 arg-10; pe*+ *fl*+; *A* × *gul-4 nt nic-3*+ *arg-10*+; *pe fl; a*
gul-4+ *nic-3 arg-10; inos*+; *pe*+ *fl*+; *A* × *gul-4 nic-3*+ *arg-10*+; *inos; pe fl; a*
gul-5+; *inos*+; *nic-3; pe*+ *fl*+; *A* × *gul-5; inos; nic-3*+; *pe fl; a*
nt, *nic-3* and *arg-10* are in VII; *pe* and *fl* are in II; *inos* is in V; *A/a* in I. Random ascospores were plated and exceptional-progeny gullivers isolated. Conidia from the EPG cultures were plated and several *gul* and *gul*+ colonies isolated from each EPG. Conidia from these were in turn plated on adequately supplemented media in order to ascertain homocaryosis for gulliver and to score other markers.

on another chromosome was detected. This might have resulted from contamination on the isolation plate. Table 4 also shows as a control the genotypes of EPGs derived from *gul-5*, a gene not linked to any of the other markers used. In this case, only two instances of marker mosaicism were recorded.

As an alternative to the pseudowild-type hypothesis, the possibility was considered that exceptional progeny gullivers might result from new mutations. This is not consistent with the results presented above. Furthermore, the spontaneous forward mutation rate is too low to account for the occurrence of EPGs (Table 5). Then, when the *gul* components of 109 EPGs were isolated and crossed again to *gul*+ in order to test their ability to segregate, 103 tested as nonsegregating and only six were segregating alleles, a ratio quite different from that encountered with gullivers obtained by mutation (11 NS to 44 segregating mutants). Finally, tests for allelism between 42 EPGs and their gulliver parents indicated agreement in every case.

DISCUSSION

The six different gulliver loci identified so far are spread throughout the genetic map. Four have been located on linkage groups IV, V, VI and VII, and the other two are as yet unmapped. The genetic behaviour of all six are quite ortho-

TABLE 5

Incidence of gullivers in crosses involving nonsegregating gullivers

Crosses*	Progeny		
	gul	*gul*+	Percentage *gul*
gul+ × NS *gul*	335	85700	0.39
gul+ × *gul*+	1	75700	0.001

* Results from 57 tests and six control crosses were pooled. In the latter, one parent was the same used in the test series; the other parent was a strain from which the nonsegregating *gul* mutants originated in a single step.

dox, even though mutants at four of the loci are not recovered from crosses because of ascospore lethality. Gullivers, defined as modifiers of *cot*, have not been studied by other authors. However, some of the pleiotropic effects of these mutants might have been identified previously as markers—in particular, ascospores lethality and morphological effects at 25°C. Thus, MURRAY and SRB (1961) located an ascospore-lethal colonial mutant (*col-le-2*) 14 map units distal from *cot*. This location is not significantly different from that reported here for *gul-3*, an ascospore-lethal gulliver, which is not colonial at 25°C. PERKINS (personal communication) has located *gul-1* between *inos* and the centromere. Three morphologicals, spray (*sp*), mad (*md*) and shallow (*sh*) are known to map in that general region, some 3 to 6 units apart (DURKEE, SUSSMAN and LOWRY 1966). The location of *gul-1* in relation to the former mutants has not yet been determined.

The relationship of the gulliver mutants to differentiation and growth regulation is of particular interest. In Neurospora, growth is confined to hyphal tips. WARD and CIURYSEK (1962) reported that younger hyphae contain actively dividing nuclei, while nuclei in the older regions are unlikely to divide. It should be noted, however, that differentiation of hyphae into growing and nongrowing regions is not a static phenomenon, because protoplasmic streaming moves cell material from the older regions toward the growing tips (ZALOKAR 1959). Besides this regional differentiation exhibited by wild-type hyphae, colonial mutants can switch the whole mycelium to the nongrowing state at a certain stage of growth. In the *cot* strains this switch is conditioned by high temperature and its timing is markedly altered in the presence of gulliver mutants.

The data available do not allow identification of the regulatory circuit(s) affected by the *gul* mutants. Certain features, however, are worth noting: (a) At least six loci yield the gulliver phenotype; (b) some of these loci have pleiotropic effects on other morphogenetic processes: the germination of ascospores, the formation of functional protoperithecia and heterocaryons, and the production of conidia; (c) in some cases, mutation to a larger colony is accompanied by temperature-independent colonial growth habit; (d) heterocaryons or pseudowild types containing *gul* and gul^+ alleles yield small colonies at first, but later burst into gulliver-like growth. Observation (d) might simply reflect a threshold effect in gene expression, such that the change from gul^+ to *gul* phenotype, due either to the accumulation of substances or to a change in nuclear ratios, is punctuated by a discontinuity. A simpler interpretation would be that *gul* and gul^+ nuclei behave autonomously, and that the latter can outgrow the former after escape from the compact colony. This interpretation is of particular interest in connection with the nature of the regulatory circuits altered by mutation. Since heterocaryons between biochemical mutants usually behave nonautonomously (PITTENGER and ATWOOD 1956), it may be assumed that diffusible metabolites are easily transported along the mycelium. Consequently the gulliver mutants, if truly autonomous, are likely to be involved in the control of nondiffusible elements of the regulatory circuits.

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SUMMARY

Thirty-three modifiers of the temperature-sensitive colonial gene *cot* were studied. These modifiers, called gulliver (*gul*), delay the onset of the stationary phase of growth and thus determine larger colonies. Six different *gul* loci were identified, and four of them mapped. The rate of forward mutation at *gul-1* is particularly high. All mutants at *gul-3*, *gul-4* and *gul-6* are infertile as females, unable to make heterocaryons, and lethal to the ascospores. A few ascospores bearing these mutants produce viable progeny; these originate as pseudowild types and are heterocaryotic for *gul* and *gul*$^+$. Mutants at *gul-1* and *gul-2* are viable in ascospores, fully fertile and heterocaryon positive, while the one mutant studied at *gul-5* is ascospore lethal, fully fertile and heterocaryon positive. This material may be useful for studies on differentiation.

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