FURTHER EVIDENCE FOR LACK OF GENE EXPRESSION IN THE TETRAHYMENA MICRONUCLEUS

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ABSTRACT

Certain *galA* mutations in the ciliated protozoan *Tetrahymena thermophila* confer an almost total loss of galactokinase activity in homozygotes. Heterokaryons have been constructed that are homogeneous for the *galA1* mutation in the ($45n$) macronucleus, but which contain a *galA*+ ($2n$) micronucleus. Soluble cell extracts prepared from these heterokaryons have been assayed for galactokinase activity, using a radiometric assay for the conversion of galactose to galactose-1-phosphate (gal-1-P). No galactokinase activity attributable to the micronuclear genes is observed in such heterokaryons. These results, obtained with the *galA1* marker, provide the first direct, quantitative evidence for the lack of micronuclear (germ line) gene expression in Tetrahymena during vegetative growth, and substantiate the predictions of previous phenotypic observations on heterokaryons and autoradiographic studies of micronuclear RNA synthesis. The generality of this conclusion will be established in the future when other enzymically assayable mutations become available for similar studies.

THE typical ciliate Tetrahymena contains two distinctly different nuclei: a small ($2n = 10$) germinal micronucleus and a large ($45n$) somatic macronucleus. The two different nuclei normally develop from a common diploid fertilization nucleus during conjugation; thus, the new micro, and macronucleus normally start out with identical genetic information (SONNEBORN 1975). Several indirect lines of evidence support the belief that the ciliate micronuclear genes are not expressed during vegetative growth (reviewed by GOROVSKY 1973). Tetrahymena strains have been constructed in which the micronucleus and the macronucleus contain different genetic markers (heterokaryons) (ALLEN 1967a, b; BRUNS and BRUSSARD 1974). In all cases tested, these heterokaryons expressed the genotype of the macronucleus and not that of the micronucleus. However, since the minimum number of copies of a dominant or co-dominant allele required to produce a given phenotype has not been determined in any case, it is possible that full expression of the two copies present in the micronucleus was simply insufficient to affect the phenotype.

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Attempts have been made to detect RNA synthesis in the micronucleus of Tetrahymena, using cytochemical and radioautographic techniques. GOROVSKY and WOODARD (1969) found that micronuclear DNA has less than 1% of the specific activity of macronuclear DNA in RNA synthesis, although MURTI and PRESCOTT (1970) presented evidence that the micronucleus appears to incorporate radioactive RNA precursors around the time of cell division. However, since cell division is coincident with the DNA synthetic period for the micronucleus (PRESCOTT and STONE 1967; GOROVSKY 1973), it has not been ruled out that the newly synthesized RNA observed by the latter group simply primes the synthesis of daughter DNA strands and that the micronucleus is otherwise transcriptionally inert.

We have sought a more direct test of the idea that the micronucleus is inactive during vegetative growth by measuring the biochemical expression of one particular gene when the wild-type allele is present only in the micronucleus. *galA* was the first Tetrahymena gene for which an assayable enzymatic defect has been identified. Tetrahymena cells homozygous for the *galA1* mutation have less than 5% of the wild-type level of galactokinase (ATP: D-galactose-1-phosphotransferase, EC 2.7.1.6), the enzyme that catalyzes the first step in the utilization of this sugar. These homozygous gal$^-$ cells are phenotypically resistant to killing by the galactose analog 2-deoxygalactose (dgal), presumably because they lack sufficient galactokinase activity to generate toxic amounts of dgal-1-phosphate and its metabolites (ROBERTS and MORSE 1978, 1980). The radiometric galactokinase assay measures the conversion of galactose to galactose-1-phosphate (gal-1-P) and is sensitive enough to detect apparent residual activity levels below that which renders the cells sensitive to killing by dgal. We have constructed several phenotypically dgal-resistant heterokaryon strains (homogeneous for *galA1* in the macronucleus, and *galA*$^+$/*galA*$^+$ in the micronucleus), and have assayed the galactokinase activity of their soluble cell extracts relative to that of extracts from gal$^-$ (dgal-R) homokaryons, as well as gal$^+$ (dgal-S) wild-type cells. We find no significant difference in galactokinase levels between the dgal-R homokaryons and heterokaryons, supporting the idea that, at least for *galA*, the micronuclear genes are not actively expressed in vegetative Tetrahymena cells.

MATERIALS AND METHODS

Cells: The clones employed were derived from inbred strain B of *Tetrahymena thermophila* [formerly *T. pyriformis*, syngen 1 (NANNEY and MCCOY 1976)] and are described in Table 1.

Routine genetic procedures: Detailed descriptions of the routine genetic methods used have been published (ORIAS and BRUNS 1975; ORIAS and HAMILTON 1979).

Growth conditions and media: For enzyme assays, 10 or 15 ml cultures were grown without shaking in 100 × 15 mm sterile plastic petri dishes at 30°, in either 2% proteose peptone supplemented with 10 μM $FeCl_3$ (PP210), or in a minimal defined medium in which cell growth is strongly stimulated by carbohydrate supplements (ROBERTS and MORSE 1980). The defined medium contained amino acids, vitamins, guanosine, uracil, trace metals and salts, as described by ROBERTS and MORSE, with the exception that a reduced amount of $FeCl_3$ (100 μM) was included. The carbohydrate source in the defined medium was 1% (w/v) glycerol, in order to obtain maximum galactokinase activity (ROBERTS and MORSE 1978). In all but one of the experiments reported in this study, the cells were grown in proteose peptone for reasons of con-

TABLE 1

List of clones used

Clone	Micronuclear genotype	Macronuclear phenotype	Mating type	Reference
BIII	wild type	wild type	III	ORIAS and HAMILTON (1979)
CU324	*Mpr-1/Mpr-1*	6mp-S	IV	ORIAS and HAMILTON (1979)
CU325	*Mpr-1/Mpr-1*	6mp-S	V	ORIAS and HAMILTON (1979)
CU329	*ChxA2/ChxA2*	cycl-S	II	ORIAS and HAMILTON (1979)
CU330	*ChxA2/ChxA2*	cycl-S	IV	ORIAS and HAMILTON (1979)
SB100	*ChxA2/ChxA2*	cycl-S, CAM-R	II	ORIAS and HAMILTON (1979)
SB210	*galA1/galA1*	dgal-S	VI	This study
SB220	*ChxA2/ChxA2*, *Mpr-1/Mpr-1*	cycl-S, 6mp-S, CAM-R	II	ORIAS and HAMILTON (1979)
dgal-R Heterokaryons				
SB230	*ChxA2/ChxA2*, *Mpr-1/Mpr-1*	cycl-S, 6mp-S, dgal-R, CAM-R	VII	This study
SB231	*ChxA2/ChxA2*, *Mpr-1/Mpr-1*	cycl-S, 6mp-S, dgal-R	IV	This study
SB232	*ChxA2/ChxA2*, *Mpr-1/Mpr-1*	cycl-S, 6mp-S, dgal-R	IV	This study
SB233	*ChxA2/ChxA2*, *Mpr-1/Mpr-1*	cycl-S, 6mp-S, dgal-R, CAM-R	VI	This study
SB506	*ChxA2/ChxA2*, *Mpr-1/Mpr-1*	cycl-S, 6mp-S, dgal-R	II	This study
SB507	*ChxA2/ChxA2*, *Mpr-1/Mpr-1*	cycl-S, 6mp-S, dgal-R	IV	This study
SB508	*ChxA2/ChxA2*, *Mpr-1/Mpr-1*	cycl-S, 6mp-S, dgal-R	IV	This study
SB237	*ChxA2/ChxA2*	cycl-S, dgal-R	II	This study
dgal-R Homokaryons				
SB234	*galA1/galA1*	dgal-R	VI	This study
SB235	*galA1/galA1*	dgal-R	VII	This study
SB236	*galA1/galA1*	dgal-R	IV	This study
SB238	*galA1/galA1*	dgal-R	VI	This study
SB509	*galA1/galA1*	dgal-R	II	This study

Chx (ROBERTS and ORIAS 1973b; BYRNE, BRUNS and BRUSSARD 1978) and *Mpr* (BYRNE, BRUNS and BRUSSARD 1978) are dominant mutations conferring resistance (R) to cycloheximide (cycl) (15 μg/ml) and 6-methylpurine (6mp) (15 μg/ml), respectively. *galA1* was used as a dominant mutation; *galA1* homozygotes and most heterozygotes are resistant to killing by 2-deoxygalactose (dgal) after about 13 fissions in nonselective medium (ROBERTS and MORSE 1980). CAM is a cytoplasmic mutation, probably mitochondrial, which confers resistance to killing by chloramphenicol (ROBERTS and ORIAS 1973a).

venience (faster growth rates and higher yields than in the defined medium); cultures in the mid- to late-exponential phase of growth in PP210 have approximately equivalent galactokinase levels as comparable cultures in the minimal defined medium (unpublished). Nutrient medium used in pair isolations in the genetic procedures was 1% proteose peptone containing either 5 or 10 μM $FeCl_3$. All the work was done under axenic conditions. Penicillin and streptomycin (Sigma) (250 μg/ml each) were added to all media to minimize the risk of bacterial contamination.

Induction of pronuclear fusion failure: dgal-R homokaryons and heterokaryons were derived from SB210 × SB220 and SB210 × SB100 crosses by inducing pronuclear fusion failure during conjugation (HAMILTON, SUHR-JESSEN and ORIAS, unpublished) as follows. Mating cultures (pre-

pared as described in ORIAS and HAMILTON 1979) were treated with 2.0 or 5.0 μg/ml of vinblastine sulfate (VBL; Sigma #V1377) at 5, 5.5 or 6 hr after mixing the cultures of two different mating types. After 30 min of incubation with VBL at 30°, the cultures were diluted 1:20 with Dryl's phosphate buffer (DRYL 1959), and single pairs were isolated into drops of nutrient medium. The isolated pairs were left at room temperature for approximately 12 hr, during which time the exconjugants separated, but did not yet divide. After the exconjugants separated, one of them was then isolated to a separate drop.

To detect fusion-failure candidates, each exconjugant clone was tested for its resistance (R) or sensitivity (S) to 2.5 mg/ml dgal, 15 μg/ml cycloheximide (cycl), 250 μg/ml chloramphenicol (CAM), and (if appropriate) 15 μg/ml 6-methylpurine (6mp). The cultures were first replica-plated to separate Microtiter® plates containing CAM, cycl and dgal, respectively. After three days, the cycl-R, and the dgal-R clones were replica-plated to the reciprocal drug (*i.e.*, cycl to dgal, and *vice versa*) and after 3 or 4 days were scored for their R or S to the second drug.

Test crosses of presumptive fusion failure progeny: In order to identify homo- and heterokaryons, *i.e.*, to determine the micronuclear genotype, testcrosses of potential fusion-failure candidates were performed. Single cells were isolated from dgal-R, cycl-S candidate clones from the first dgal plate, were taken to maturity, and their mating type was then determined. They were then testcrossed to strains of known genotype and phenotype (to CU325, CU329 or CU330, and in some cases to BIII).

In the selection of potential fusion-failure strains to be testcrossed, preference was given to CAM-S clones wherever possible, and an attempt was made to maximize the variety of mating types. In the selection of strains to be assayed for galactokinase, preference was given to clones having good viability and fertility in the testcrosses, and whose testcross results were clear and unambiguous.

Preparation of cell extracts: Mid- to late-exponential phase cultures were harvested for enzyme assays. Proteose peptone cultures were harvested at betwen 3.1 to 7.5×10^5 cells per ml; they can reach densities of over 1×10^6 cells/ml under these conditions. Defined media cultures were harvested at between 5.3 and 9.7×10^4 cells/ml; they can reach a maximum of about 1.1×10^5 under these conditions. 3×10^5 cells were harvested per sample of phenotypically dgal-S culture, and 10^6 cells were harvested per sample of phenotypically dgal-R culture, when grown in either medium.

Cells were harvested by centrifugation in 15 ml conical centrifuge tubes at room temperature for 1 min at about $500 \times g$ in an International Clinical centrifuge. The cell pellets were washed and resuspended in a Tris/dithiothreitol buffer, as described by (ROBERTS and MORSE (1978). The cell suspensions were sonicated, and soluble cell extracts were also prepared as described by those authors. The extracts were immediately assayed for galactokinase activity and were then frozen until it was convenient to assay for protein concentration. Protein content was determined by the method of BRADFORD (1976).

Galactokinase assay: Galactokinase activity was assayed by measuring the amount of radioactive galactose converted to gal-1-P in 45 minutes at 37°. The reaction was carried out as described by ROBERTS and MORSE (1978, 1980), with the following exceptions: the labeled sugar used was ^{14}C-D-galactose (0.5 μCi/ml), a 45-min incubation period was used and the final reaction mixture had a total volume of 50 μl, including up to 25 μl of cell extract. Enzyme units are expressed as nmoles gal-1-P produced per hr. Enzyme specific activity is expressed as enzyme units per mg protein and was determined from the least-squares slope for the graph of enzyme units *vs.* mg of protein in the reaction mixture. Under these conditions, the amount of gal-1-P produced was directly proportional to the time of incubation and to the amount of cell extract protein in the reaction mixture.

Clones homozygous for the *galA1* mutation phosphorylate D-galactose with 3 to 5% of the wild-type specific activity. This residual activity represents an enzymatic phosphorylation of ^{14}C-D-galactose: the reaction product is sensitive to bacterial alkaline phosphatase (unpublished data) and is not formed if the cell extract is first heat treated at 90° for 3 min (see RESULTS).

Reagents: Chemicals were obtained from the following sources: D-galactose (for the reaction mixture) from Pfanstiehl; Tris, EDTA, dithiothreitol, D-galactose (for washing the filters),

ATP and $MgCl_2$ from Sigma; glycerol from Baker; D-galactose [(U)^{14}C] (spec. act. 320 mCi/mmole; radiochemical purity $\geq$ 98%) from New England Nuclear.

RESULTS

Isolation and genetic characterization of dgal-R- homo- and heterokaryons: dgal-R homokaryons and heterokaryons were constructed by inducing pronuclear fusion failure during conjugation, as described by HAMILTON, SUHR-JESSEN and ORIAS (unpublished) and in MATERIALS AND METHODS. When fusion failure is induced, the migratory pronuclei are exchanged, but they fail to fuse with the stationary pronuclei. As a result, each exconjugant cell gives rise to two genetically different daughter cells (called caryonides). One caryonide is a homokaryon, with both micro- and macronucleus derived from one parental micronucleus, while the other is a heterokaryon, with the micronucleus derived from one parent and the macronucleus derived from the other. (See Figure 1 for a further explanation of the consequences of pronuclear fusion failure.) For both the heterokaryons and homokaryons, both the macro- and the micronucleus are homozygous for the whole genome (HAMILTON, SUHR-JESSEN and ORIAS, unpublished).

Presumptive fusion-failure clones from crosses of SB210 to SB220 or SB100 were identified as follows. Exconjugant cultures resulting from presumptive fusion-failure pairs should contain a mixture of two phenotypically different cell types (if both caryonides survived): dgal-S, cycl-R, and dgal-R, cycl-S, respectively; in addition, one exconjugant clone should be CAM-S, and the other CAM-R (FIGURE 1D). Sets of exconjugant clones that showed this distinctive drug-resistance pattern were considered to be excellent fusion-failure candidates. Since the occurrence of this complete drug-resistance pattern was relatively rare, consideration was also given to those cases in which only one exconjugant clone showed the expected fusion-failure pattern, as well as to those cases in which the exconjugant clone was homogeneous for dgal-R, cycl-S. (Exconjugant clones arising as a result of pronuclear fusion failure would be phenotypically homogeneous if only one of the two caryonides survives. Homogeneous dgal-R, cycl-S, CAM-S clones could have arisen either by fusion failure or by self-fertilization of the SB210 parent.)

Fusion-failure candidates were then testcrossed to identify the desired heterokaryon and homokaryons, as described in MATERIALS AND METHODS. The results are shown in Tables 2 and 3, respectively. Table 4 summarizes the number of potential fusion-failure clones falling into the three categories of phenotype pattern discussed above, the genotype determined for every testcrossed clone, and the origin of the clones chosen for the biochemical tests.

Galactokinase activity in homo- and heterokaryons: The specific activity of galactokinase in eight independently obtained heterokaryons possessing *galA*+ alleles only int he micronucleus was compared to five independently obtained, all *galA1* homokaryons. Table 5 summarizes the data obtained in six separate experiments, and Figure 2 is a graphic representation of the data obtained from a typical experiment. The mean of the specific activity measured in the hetero-

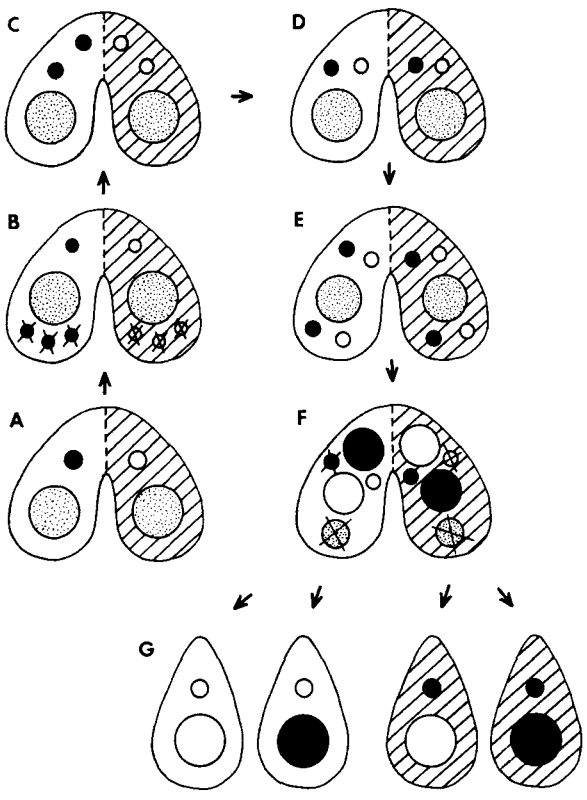

FIGURE 1.—Scheme for generating dgal-R homo- and heterokaryons by fusion failure. *Key to symbols*: Black and white circles represent nuclei homozygous for *galA1* and for *ChxA2* (or *ChxA2* and *Mpr-1*), respectively; stipled circles represent the old macronuclei, which are wild type for all the genetic loci; cross-hatching represents the CAM-R cytoplasmic marker.

Initiated cells of two different mating types fuse together at the anterior portion of the cell to form heterotypic pairs (A). The micronuclei undergo meiosis (B); one of the meiotic products in each cell divides again to form a stationary and a migratory pronucleus (C), and the migratory pronuclei are exchanged (D). When fusion failure is induced, the migratory and stationary pronuclei do not fuse to form a zygotic nucleus, as in normal conjugation; instead each separate pronucleus undergoes one mitosis to yield the characteristic four nuclear products normally seen after the second post-zygotic mitosis (E). The remainder of conjugation proceeds normally. Anterior division products differentiate into new macronuclei, while the posterior nuclear products become micronuclei (F); the old macronucleus is destroyed. The conjugants separate. One randomly selected new micronucleus is destroyed in each exconjugant; the remaining micronucleus divides mitotically. Each of the four caryonides (*i.e.*, products of the first vegetative cell division) receives one new micronucleus and one new macronucleus (G).

Genetic consequences of fusion failure: (a) each exconjugant yields one homokaryon and one heterokaryon, whose micronuclei are genetically identical (derived from one parent or the other). (b) When both caryonides survive, each exconjugant gives rise to a mixed clone. (c) Each fusion-failure caryonide carries homozygous nuclei, each derived from a single meiotic product of one parent or the other.

TABLE 2

Homokaryon testcross data

	Phenotype of F_1 progeny					
	Progeny showing evidence of cross-fertilization					
(a) Testscrosses to *Chx/Chx* heterokaryons	cycl-R					
	6mp-R		6mp-S			
	dgal-R	dgal-S	dgal-R	dgal-S	NC*	Dead
SB236 × CU329	0	0	93	0	3	0
SB238 × CU329	0	0	45	0	2	1
SB234 × CU330	0	0	82	2†	9	3
SB235 × CU329	0	0	42	0	5	1
SB509 × CU330	0	0	90	0	2	4
(b) Testscrosses to *Mpr/Mpr* heterokaryons	6mp-R					
	cycl-R		cycl-S			
	dgal-R	dgal-S	dgal-R	dgal-S	NC*	Dead
SB236 × CU325	0	0	80	0	15	1
SB238 × CU325	0	0	27	2†	10	9
SB234 × CU325	0	0	76	0	15	5
SB235 × CU325	0	0	19	0	21	8
SB509 × CU325	0	0	82	0	7	7

Crosses to *Chx/Chx* heterokaryons test for both the *gal* and *Mpr* micronuclear genotype; those to *Mpr/Mpr* heterokaryons test for both the *gal* and *Chx* micronuclear genotype. Either 48 or 96 single pairs were isolated from each cross into drops of nutrient medium. The resulting clones were first replica-plated to separate microtiter plates containing cycl, 6mp and dgal, respectively. (In some cases, the clones were replica-plated to nutrient medium in microtiter plates, and the resultant clones were used for the first drug replications). The resistant clones were subsequently replica-plated to the two other drug conditions; *e.g.*, the cycl-R clones were then replica-plated to 6mp and dgal, respectively. The micronuclear genotype inferred from the above data for all four strains was: *galA1/galA1*. (Strain SB234 has become sterile since first testcrossed, and strain SB238 has been lost due to contamination.)

* NC = nonconjugants (cycl-S, 6mp-S and dgal-R or -S).

† The occasional presence of dgal-S clones among the presumptively heterozygous testcross progeny is due to insufficient phenotypic assortment (ROBERTS and MORSE 1980).

karyons (17.5 ± 0.9) was not significantly higher than the residual activity seen in the homokaryons (15.9 ± 0.8) (Table 5).

This lack of significance in the difference between the hetero- and homokaryons was determined in three ways. (1) A Student t test for significance of the difference between the mean of the specific activities of the five homokaryons on the one hand, and the mean of the specific activities of the eight heterokaryons on the other, gave a negative result ($P > 0.2$). (2) The least-squares slope was computed for the combined heterokaryon data points (17.4 ± 1.4) and for the combined homokaryon data points (15.2 ± 1.4) for the experiment shown in Figure 2. The null hypothesis that the two regression lines have equal slopes was tested; the computed t-test statistic (1.09) fell within the 95% confidence interval for 20 degrees of freedom ($-2.086 < t < +2.086$). (3) The coefficient of determination (r^2) of the least-squares slope, fit to *all* the points in Figure 2 ($r^2 = 0.928$), is close to the average of the coefficients of the two lines separately fit to all the heterokaryon points ($r^2 = 0.941$) and to all the homokaryon points ($r^2 =$

TABLE 3

Heterokaryon testcross data

Cross	Phenotype of F_1 progeny: Progeny showing evidence of cross fertilization cycl-R†					
	6mp-R		6mp-S‡			
	dgal-R	dgal-S	dgal-R	dgal-s	NC*	Dead
SB230 × BIII	0	87	0	1	5	3
SB230 × CU329	0	90	0	2	2	2
SB230 × CU325	0	81	0	0	13	2
SB231 × BIII	0	83	0	1	11	1
SB231 × CU329	0	84	0	0	11	1
SB231 × CU325	0	89	0	0	5	2
SB232 × BIII	0	86	0	1	6	3
SB232 × CU329	0	91	0	1	2	2
SB232 × CU325	0	85	0	0	9	2
SB233 × BIII	0	87	0	0	5	4
SB233 × CU329	0	86	0	1	4	5
SB233 × CU325	0	77	0	0	13	6
SB508 × BIII	0	92	0	1	1	2
SB508 × CU329	0	92	0	0	2	2
SB508 × CU325	0	88	0	0	8	0
SB506 × BIII	0	85	0	7	2	2
SB506 × CU330	0	80	0	9	2	5
SB506 × CU325	0	80	0	0	14	2
SB507 × BIII	0	73	0	4	12	7
SB507 × CU329	0	83	0	2	10	1
SB507 × CU325	0	66	0	0	24	6
SB237 × BIII	0	0	0	94	2	0
SB237 × CU325	0	78	0	2	13	3
SB237 × CU330	0	0	0	92	2	2

96 single pairs were isolated from each testcross into nutrient medium. The phenotypes of the testcross progeny were determined, as described in the legend to Table 3. The genotype deduced for SB237 was *ChxA2/ChxA2*; the genotype deduced for all the other strains was *ChxA2/ChxA2, Mpr-1/Mpr-1*.

* NC: see Table 2.

† No cycl-S, 6mp-R progeny were observed.

‡ The reason for the presence of a low, but consistent percentage of cycl-R, 6mp-S clones among the predominantly cycl-R, 6mp-R progeny clones is presently undetermined. Extensive phenotypic tests and testcrosses failed to reveal any copies of the *Mpr-1* allele in either their macro- or micronucleus.

0.920), respectively. Thus, the fit of the single line is no worse than the fits of the two separate lines to each of the two classes of points. Thus, no galactokinase activity attributable to the $galA^+/galA^+$ micronucleus of the dgal-R heterokaryons can be detected, supporting the idea that the micronucleus is inert or much less active than the macronucleus with regard to gene expression.

An important assumption behind our conclusion is that mutant polypeptides specified by the macronucleus do not form mixed oligomers with, and inactivate, putative wild-type polypeptides produced by the micronucleus. To look for evidence of such an interaction, we assayed the galactokinase specific activity in $galA1/galA^+$ heterozygotes shortly after their formation by conjugation be-

TABLE 4

Summary of analysis and disposition of fusion-failure candidates

Class of fusion-failure candidate	Sets observed	Clones testcrossed	Results			Origin of clones used
			Homozygous homokaryons	Homozygous heterokaryons	Other	
A. Complete set of all four caryonides	7					SB231, SB233,
(1) dgal-R, cycl-S clones		11	3	8	—	SB506, SB507, SB508, SB509
B. Only one complete exconjugant set:	27					
(1) dgal-R, cycl-S clone from the complete set.		10	4	4	2*	SB232, SB234, SB235, SB237
(2) dgal-R, cycl-S clone from the incomplete exconjugant set.		5	5	—	—	
C. Homogeneous dgal-R, cycl-S exconjugant (xc) clone.					—	
(1) both xc clones dgal-R	18	5	2	3		SB230
(2) only one xc clone dgal-R	106	10	9	1		SB236, SB238

These data represent the combined results of four separate experiments, in which a total of 888 single pairs were isolated. Genotypes were determined by testcrosses (Tables 2 and 3).

* One clone had a heterozygous micronucleus, formed by cross fertilization; the other clone was sterile, *i.e.*, did not produce viable testcross progeny.

TABLE 5

Galactokinase specific activity (nmoles gal-1-P/hr mg protein) in various homo- and heterokaryons

Strains	Number of experiments	Mean specific activity ± 1 standard error of the mean	Average r^2 value*
dgal-S			
BIII	6	541.3 ± 27.1	0.994
CU329	5	574.7 ± 110.6	0.966
CU324	3	544.2 ± 37.9	0.991
SB100	1	357.6	0.999
SB220	1	540.7	1.000
SB210	3	454.7 ± 69.6	0.997
		mean 502.2 ± 33.3	
dgal-S homozygous homokaryons			
F_1 (SB232 × SB230)			
clone 1–1G	2	556.5 ± 13.5	0.996
clone 1–2B	2	614.3 ± 67.8	0.994
clone 1–2H	2	579.0 ± 59.1	0.999
clone 1–3B	2	300.0 ± 20.1	0.978
clone 1–3D	1	556.8	0.987
clone 1–3F	1	530.4	0.947
		mean 522.8 ± 46.0	
dgal-R homozygous homokaryons			
SB234	4	16.3 ± 1.3	0.987
SB235	2	16.8 ± 0.6	0.994
SB236	4	18.0 ± 0.6	0.952
SB238	1	14.3	0.998
SB509	3	14.1 ± 0.9	0.978
		mean 15.9 ± 0.8	
dgal-R homozygous heterokaryons			
SB230	5	17.0 ± 2.1	0.952
SB231	3	16.8 ± 0.6	0.989
SB232	5	17.6 ± 1.9	0.968
SB233	4	22.4 ± 3.4	0.979
SB237	1	15.4	0.993
SB506	2	15.8 ± 2.3	0.959
SB507	1	20.2	0.969
SB508	1	14.9	0.940
		mean 17.5 ± 0.9	

The data in this table are the pooled results of six independent experiments. Each cell extract was assayed for enzyme activity at 3 to 5 different protein concentrations, ranging from 1.1 to 15.3 μg per reaction mixture for extracts prepared from dgal-S cells, and 5.3 to 47.5 μg per reaction mixture for dgal-R cell extracts. In five of the experiments, extracts were prepared from cultures grown in PP210 medium and, in one experiment, the cultures were grown in defined medium; the results were comparable for both media.

* r^2 = linear correlation coefficient for the least-squares slope of the graph of enzyme units *vs.* mg protein in the reaction mixture.

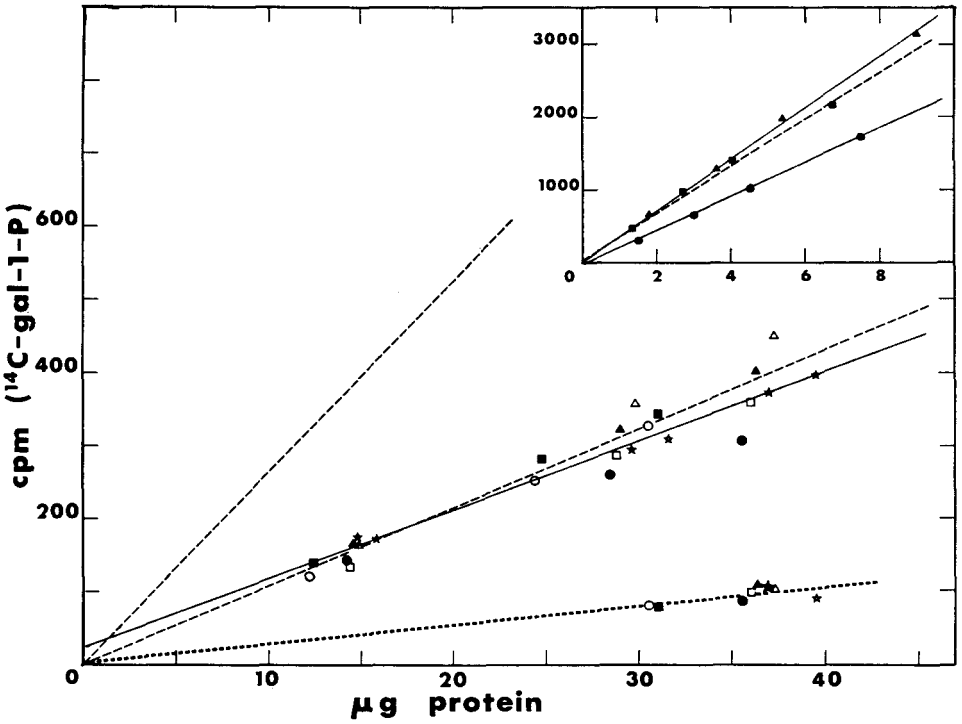

FIGURE 2.—Comparison of galactokinase activity of cell extracts prepared from dgal-R homokaryons and heterokaryons, and dgal-S homokaryons.

Ordinate: Counts per minute of ^{14}C-gal-1-P (180 cpm background was subtracted from each sample); *abscissa*: μg of cell extract protein (prepared from exponentially growing cultures in PP210 medium) in the reaction tube. Incubation time was 45 min at 37°; specific activity of the ^{14}C-D-galactose in the reaction mixture was 5.0×10^{-4} μCi/nmole; counting efficiency was 75.1%. Open symbols are data for dgal-R heterokaryon strains; the dashed line running through them is their least-squares slope. Closed symbols are data for dgal-R homokaryons, and the solid line is their least-squares slope. The heavy dashed line runs through the data obtained when the cell extracts were heat killed (3 min at 90°) prior to incubation with the reaction mixture. The upper dashed line represents the enzyme units as a function of cell extract protein expected for a dgal-R heterokaryon if micronuclear *galA*+ alleles were expressed at the same level as macronuclear ones. This line was calculated using the following assumptions (see text): (1) galactokinase activity is directly proportional to gene dosage; (2) the ratio of macronuclear to micronuclear *galA* gene copies is 17, when averaged over an asynchronously growing population; (3) inactive polypeptides specified by the *galA1* allele do not inactivate wild type (*galA*+) polypeptides; (4) the putative enzymatic activity from the micronucleus is additive to the residual activity seen in a dgal-R homokaryon. The mean specific activity (479.4 ± 49.4) of the three dgal-S strains (inset) was used to calculate the expected slope. This slope differs from that generated by all the heterokaryon points at the 1% significance level.

Key to symbols: △ SB230, □ SB231, ○ SB232, ★ SB233, ● SB234, ▲ SB235, ■ SB236, ★ SB238.

The inset shows the enzymic activity of three dgal-S wild-type strains assayed in the same experiment. The abscissa and the ordinate units are the same as those in the main graph. Key to symbols: ● CU329, ○ CU234, △ BIII.

tween a dgal-R homokaryon and a dgal-R heterokaryon. These activities were compared to the galactokinase specific activities measured in parallel *galA+/galA+* (dgal-S) homokaryon progeny clones derived from a control cross of two heterokaryons to each other. The heterozygous clones generally show intermediate levels of enzyme activity (Table 6); the mean for all the heterozygous progeny tested was 62% that of the dgal-S wild-type homokaryon progeny, although there was a wide range of specific activities, from a low of 30% to a little over 100% of the wild-type controls. Thus, we find no evidence that the activity of the wild-type polypeptides is diminished in the presence of mutant polypeptides. (The wide range of specific activities of individual F_1 clones can be at-

TABLE 6

Galactokinase specific activity (nmoles gal-1-P/hr mg protein) in galA1/galA+ *heterozygous and wild-type dgal-S homozygous clones*

	Specific activity	r^2
F_1 (SB230 × SB232) (*galA+/galA+*, dgal-S homozygous homokaryons)		
clone A7G	561.3	0.974
clone A7H	655.1	0.998
clone A9D	430.5	0.991
clone A9H	533.2	0.996
clone A10H	276.3	0.989
clone A11A	437.9	0.991
	mean 482.4 ± 53.5	
F_1 (SB232 × SB509) (*galA1/galA+* heterozygotes)		
clone B1C	497.4	1.000
clone B2B	262.9	0.967
clone B3D	258.7	0.986
clone B4C	268.1	0.984
clone B5C	267.1	0.987
clone B7H	271.8	0.941
	mean 304.3 ± 38.7	
F_1 (SB230 × SB236) (*galA1/galA+* heterozygotes)		
clone C1E	145.0	0.989
clone C4B	436.8	0.973
clone C5C	425.8	0.988
clone C6G	252.3	0.977
clone C7G	227.7	0.976
	mean 297.5 ± 57.5	

Mean for all heterozygous progeny: 301.2 ± 31.8

Each cell extract was assayed for enzyme activity at 3 or 4 different protein concentrations, ranging from 2.3 to 32.1 μg per reaction mixture for extracts prepared from dgal-S cells, and 13.0 to 44.8 μg per reaction mixture for dgal-R cell extracts. Cell extracts were prepared from cultures grown in PP210 medium. F_1 progeny clones were assayed for galactokinase activity 13 days after the cross was performed, although they were kept under conditions in which we estimate they went through fewer than 28 fissions.

tributed to the known variability in the ratio of wild-type to mutant allele copies established during macronuclear differentiation; NANNEY *et al.* 1963). Furthermore, assays of mixtures of extracts from wild-type and dgal-R cells failed to indicate the presence of galactokinase inhibitors or activators (C. ROBERTS, personal communication; our unpublished results). The results of both the heterozygote studies and the mixing experiments are consistent with the assumption that the level of enzyme activity is roughly proportional to the number of *galA*+ allele copies. This conclusion applies to the *galA* gene expression, regardless of whether it is hte structural gene for galactokinase (as seems likely; ROBERTS and MORSE 1980) or specifies a positive regulator of galactokinase synthesis.

DISCUSSION

Our studies with dgal-R heterokaryons failed to detect any statistically significant galactokinase activity attributable to the expression of *galA*+ genes in the micronucleus. These findings, using an enzymatically assayable phenotype, support previous conclusions derived from phenotypic and autoradiographic observations, summarized above, which suggest the total lack of gene expression in the micronucleus. Mutations causing loss-of-activity alleles for other assayable enzymes in Tetrahymena are rapidly accumulating (thymidine kinase. MARTINDALE and PEARLMAN 1979; glucokinase, ROBERTS, LAVINE and MORSE 1980; phenylalanine hydroxylase, SANFORD and ORIAS 1981) and will permit a test of the generality of our observations. Meanwhile, we are using the dgal-R heterokaryons to investigate the stage in conjugation when genes in the differentiating macronucleus become active (MAYO and ORIAS 1979).

The sensitivity of our (negative) findings can be evaluated by the following considerations. Taking into account the variability of the data presented in Table 5, we estimate that the observed heterokaryon mean would have to be at least 19.2 before it would significantly differ from the homokaryon mean at the 95% confidence level. Even if the actual heterokaryon mean were 19.6 (upper 95% confidence limit of the observed mean), the overall level of micronuclear *galA*+ expression would still be at most 1% of the overall macronuclear level; given the approximate 17-fold ploidy ratio (on the average)[1] between the two nuclei, each micronuclear copy must be expressed at less than 15% of the level of each macronuclear gene copy. Even a two-fold error in these estimates would not alter the basic conclusion that the micronuclear alleles are expressed at a significantly lower rate than macronuclear gene copies, if at all.

[1] We estimate a total of approximately 67 allelic copies of the *galA* gene per cell, averaged over all cells in an asynchronously growing population: 3.8 copies per micronucleus and 63.2 copies per macronucleus. These numbers are based on cytofluorometric analysis of DNA in the two nuclei (DOERDER and DEBAULT 1975), on the timing of DNA replication in the two nuclei during the cell cycle (MCDONALD 1962; WOODWARD, KANESHIRO and GOROVSKY 1972; see GOROVSKY 1973, for the macronucleus), on assortment rates in heterozygotes (reviewed by NANNEY and PREPARATA 1979), and on the relative kinetic complexity of macronuclear and micronuclear DNA (YAO and GOROVSKY 1974). We have also assumed that, in terms of copy number, the *galA* gene behaves like every gene in *T. thermophila* whose assortment rates have been measured (average of 45 copies per G1 macronucleus). Extensive experience in our lab with routine isolation of dgal-S assorters suggests that this assortment rate, while not carefully measured, does not qualitatively differ from that measured with other genes.

One possible problem with our conclusions is that we may have inadvertently biased the results by using dgal-R as a criterion in isolating the heterokaryons, thus possibly insuring our failure to detect micronuclear gene expression. (In hindsight, it would have been preferable to detect fusion-failure progeny without reference to the dgal phenotype; the present clones were originally isolated for a different purpose, for which this consideration was irrelevant. We are isolating and assaying new heterokaryons, using genetic criteria independent of the dgal phenotype.) Nevertheless, we strongly believe that the strains isolated are representative heterokaryons for the following reasons: First, they were obtained by a process with well-defined and predictable genetic consequences (fusion failure), documented by the behavior of other markers (*ChxA1*, *Mpr-1*). Furthermore, the heterokaryons have well-established phenotypic and genotypic behavior Tables 3, 4). The existence of a second class of heterokaryons with identical genotype but hereditarily expressing a different (fully dgal-S) phenotype would be totally unexpected. Second, this possibility seems unlikely on empirical grounds. From our work, we know that homozygous clones having residual galactokinase levels as high as 6% of the activity in wild-type clones are still fully resistant to dgal. Expression of micronuclear *galA*$^+$ alleles at full macronuclear levels should theoretically confer only about 10% of wild-type activity, and it seems very unlikely that such a small increment would confer full dgal sensitivity.

The observation that two different, remarkably stable states of gene expression are maintained by the two nuclei in a common cytoplasm and in close physical juxtaposition presents an interesting problem in eukaryotic developmental genetics. Except during the short period of micronuclear division, the micronucleus physically resides in a cup-like invagination of the macronucleus (FLICKINGER 1965). Although normally the two nuclei maintain independent double bilayer membranes, occasional electronmicrographs show the two nuclei sharing the outer bilayer (ELLIOTT and KENNEDY 1973).

The molecular and biochemical basis for the maintenance of the inertness of the micronucleus in such close proximity to the actively expressed macronucleus is at present unknown. Differences have been observed between the histones of the micronucleus and those of the macronucleus, including differences in methylation and acetylation of specific histone proteins, as well as differences in the actual species of histone proteins present in the two nuclei (see review by GOROVSKY 1973; JOHMANN and GOROVSKY 1976; GLOVER *et al.* 1979). Furthermore, in DNA isolated from macronuclei, 0.65 to 0.80% of the adenine bases are methylated; whereas, the level of methyladenine in micronuclear DNA is at least 10-fold lower (GOROVSKY, HATTMAN and PLEGER 1973). Self-perpetuating differences in histone and DNA methylation may play a role in establishing and maintaining the two different states of gene expression; if so, it is interesting to ask how the differential accessibility of histones and other proteins to the DNA of the two nuclei is controlled at the time of macronuclear development. It also is known that 10 to 15% of micronuclear DNA sequences are lost from the macronucleus (YAO and GOROVSKY 1974; review by YAO and GALL 1979), and that some structural alterations of the macronuclear DNA occur (review by YAO and GALL 1979;

YAO and GALL 1976; YAO, BLACKBURN and GALL 1978), presumably at the time of macronuclear differentiation. It would be interesting if the difference in the functional state of the two nuclei ultimately turns out to be determined by programmed alterations in DNA primary sequence.

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Note added in proof: Five new heterokaryons, homozygous for dgal sensitivity in the micronucleus, and for dgal resistance in the macronucleus, constructed *without reference to the dgal phenotype*, (1) are all phenotypically dgal-R, as expected, and (2) again do not differ significantly from dgal-R homokaryons with respect to galactokinase activity.

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