Molecular and Genetic Analyses of the B Type Surface Protein Gene from *Paramecium tetraurelia*

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ABSTRACT

The gene encoding the B type variable surface protein from *Paramecium tetraurelia* stock 51 has been cloned and sequenced. The 7,182 nucleotide open reading frame contains no introns and encodes a cysteine-rich protein that has a periodic structure including three nearly perfect tandem repeats in the central region. Interestingly, the *B* gene is located near a macronuclear telomere as was shown previously for two other paramecium surface protein genes. In this paper, we characterize four independent mutants with complete macronuclear deletions of the *B* gene. Previous analysis of different macronuclear deletion mutants of the *A* surface protein gene demonstrated two types of inheritance: typical Mendelian segregation (as illustrated by d12) and cytoplasmic inheritance (shown by d48). F_1 analysis of four B^- mutants crossed with wild-type cells reveals heterozygous F_1 cell lines derived from both parental cytoplasms contain approximately the same copy number of the *B* gene, as expected for a recessive Mendelian mutation. Analysis of F_2 progeny from three of these four B^- mutant crosses indicates that one of the three exhibits a Mendelian 1:1 segregation ratio of B^+ and B^- cell lines. The other two show a preponderance of B^+ cells, but this is not correlated with the parental cytoplasmic type. In addition to having a large number of B^+ individuals, the d12.144, A^-, B^- mutant produced some F_2 progeny that stably maintain less than normal macronuclear amounts of the *A* gene and/or the *B* gene.

THE family of variable surface proteins in the ciliated protozoan *Paramecium tetraurelia* consists of at least 11 known types encoded by distinct, unlinked genes [reviewed in CARON and MEYER (1989) and PREER 1986)]. These genes encode large (251,000–308,000 daltons) glycoproteins that cover the outer surface and cilia of every cell. The proteins have been grouped into subfamilies on the basis of their antibody cross-reactivity. A, B, G and Q form one related group; D, J and M form another and C, E and H are not related to either group or to each other (PREER 1959). One of the most interesting aspects of these proteins is that they display mutually exclusive expression. That is, although a single cell line is capable of expressing a number of these proteins, only one is expressed at any given time. A cell may switch from one surface protein to another in response to environmental change. Cultures expressing a particular surface protein define a serotype, because treatments with antiserum made against a particular surface protein immobilize and kill cells that are currently expressing that protein.

Several surface protein genes have previously been cloned, including the *A*, *C*, *D* and *H* genes from *P. tetraurelia* stock 51, and the *G* gene from both stocks 156 and 168 of *P. primaurelia* (FORNEY *et al.* 1983; GODISKA 1987; SCHMIDT 1987). The *51A*, *51C*, *156G* and *168G* genes have been completely sequenced (PRAT *et al.* 1986; PRAT 1990; NIELSEN, YOU and FORNEY 1991). The characterization of these genes has permitted the molecular analysis of mutations that affect the expression of the variable surface protein genes. Some of these mutations have dramatic effects on the nuclear DNA rearrangements and processing that occur during the sexual life cycle of paramecium.

P. tetraurelia contains two different types of nuclei, the highly polyploid macronucleus, which is transcriptionally active and hence determines the phenotype of the cell, and the two diploid micronuclei, which are transcriptionally silent but participate in the sexual events of conjugation (mating) and autogamy (self-fertilization). During these events, the old macronucleus is destroyed and a new one, as well as two new micronuclei, are formed from a single zygotic nucleus. Dramatic DNA rearrangements occur during formation of the macronucleus, which lead to differences in genetic content between the two types of nuclei. Some of these differences are characterized by a pattern of non-Mendelian inheritance. Perhaps the best characterized example of this kind of difference is seen in the mutant cell line d48. Originally isolated after X-ray irradiation, this cell line lacks the A surface protein gene in its macronucleus, and is therefore unable to express the A surface protein, but it contains a normal wild-type *A* gene in its micronucleus (KOIZUMI and KOBAYASHI 1989; RUDMAN *et al.* 1991). Unlike Mendelian mutations, which normally segregate 1:1 in the F_2 generation regardless of the parental cytoplasm, a

cross between wild-type and d48 cells typically yields wild-type cells from the cytoplasm of the wild-type parent and mutant cells from the cytoplasm of the d48 parent in the F_2 generation (EPSTEIN and FORNEY 1984). A number of experiments have indicated that the d48 mutant lacks an element that is required for correct processing of the *A* gene into the new macronucleus (HARUMOTO 1986; KOIZUMI and KOBAYASHI 1989). Rescue of this mutation by microinjection of the entire *A* gene or portions thereof have indicated that the element required for correct processing of the gene into the new macronucleus is the *A* gene itself (GODISKA *et al.* 1987; YOU *et al.* 1991; JESSOP-MURRAY *et al.* 1991). Hence, once lost from the original macronucleus, the *A* gene can never be processed from micronuclear DNA into the new macronucleus without first being present in the old macronucleus. Although the molecular mechanism for such a form of regulation is entirely unknown, such a system is attractive because it provides specificity for the *A* gene trait in the simplest form possible. It is not known whether other traits that have a non-Mendelian pattern of inheritance are also a result of this phenomenon, since the genes for these traits have not been cloned.

In addition to the non-Mendelian mutant d48, mutants in the *A* gene that are inherited in a Mendelian fashion have also been isolated (EPSTEIN and FORNEY 1984; RUDMAN *et al.* 1991). One of these mutants, d12, lacks the complete *A* gene in its macronucleus like d48 (PREER 1986), but unlike d48, the d12 mutant appears to lack the *A* gene in its micronucleus as well (J. R. PREER, personal communication). Crosses of d12 to wild-type cells result in F_1 exconjugant clones that are A^+ and F_2 cell lines that are either A^+ or A^- in a 1:1 Mendelian ratio. These results are what would typically be expected from a single recessive gene mutation.

In this paper, we report the cloning, telomeric location and complete sequence of the *B* type surface protein gene from *P. tetraurelia* stock 51. We have followed the inheritance of the *B* gene in genetic crosses of wild-type cells to four different B^- macronuclear deletion mutants. Genomic Southern analysis of the *B* gene was used to determine whether these mutants inherit the *B* gene in a Mendelian fashion like d12, or a non-Mendelian fashion like d48.

MATERIALS AND METHODS

Cell lines and cultivation: *P. tetraurelia* stock 51 is homozygous for both the *A* and *B* genes. d12.59, d12.138, d12.141 and d12.144 are all homozygous A^- and B^- mutants that were generously provided by the laboratory of JOHN PREER. All of the double mutants were originally derived from the Mendelian A^- mutant d12. All cells were cultured in 0.25% wheat grass medium buffered with sodium phosphate. Medium was inoculated with a nonpathogenic strain of *Klebsiella pneumoniae* 1 or 2 days prior to being used. When cells expressing surface antigen B were desired, $ZnCl_2$ was added to the culture medium at a concentration of 10 μg per ml prior to inoculation.

Isolation of DNA and RNA from paramecium: Large scale DNA isolations were performed as follows: Packed cells (0.1–0.2 ml) from 8-liter cultures were resuspended in 0.7 ml of culture fluid and then quickly squirted into 2.1 ml of lysing solution (10 mM Tris-HCl, pH 9.5, 50 mM sodium EDTA, 1% SDS,) at 65°. After 10 min, 7 ml of saturated CsCl was added and the solution was centrifuged in a vTi65.1 rotor at 55,000 rpm for approximately 20 hr. DNA containing fractions were collected and dialysed overnight against TE. Small scale DNA isolations were performed on F_1 and F_2 cell lines as follows: 100-ml cultures of each cell line were pelleted, resuspended in 0.4 ml of culture fluid and quickly squirted into 0.8 ml of lysing solution at 65°. After 10 min, lysates were extracted with phenol: chloroform and precipitated in two volumes of ethanol. Collected pellets were resuspended in TE, treated with RNase A followed by proteinase K and then extracted twice more with phenol:chloroform before a final ethanol precipitation. RNA was isolated using a guanidine-HCl method previously described (PREER *et al.* 1981).

Library constructions: A d12 (A^-), genomic library was constructed by digesting genomic d12 DNA to completion with the restriction enzyme *Eco*RI and then ligating the *Eco*RI fragments into the vector λgtWES.λB (BRL, Life Technologies, Inc.), which was previously digested with *Eco*RI and treated with calf intestinal alkaline phosphatase. The ligated product was then packaged into phage using the Promega Packagene system (Promega, Madison, WI). A second library was constructed from wild-type *P. tetraurelia*, stock 51 total genomic DNA. After brief digestion with Bal 31 nuclease, the DNA was treated with *Bam*HI methylase and then *Bam*HI linkers were ligated to the free ends. The genomic DNA was digested with *Bam*HI nuclease (to digest linkers) and partially digested with *Sau3A*. DNA fragments between 15 and 20 kb were isolated and ligated to *Bam*HI digested EMBL4.

Hybridizations: Southern and Northern blot analyses were prepared according to MANIATIS, FRITSCH and SAMBROOK (1982). Filters were washed in 10× Denhardt's solution, 0.1% SDS, 0.2 M phosphate buffer and 5× SET (1× SET = 0.15 M NaCl, 30 mM Tris, 2 mM EDTA) at 65° for 1 hr. The filters were then incubated with hybridization solution (1× Denhardt's, 20 mM phosphate buffer, 5× SET and 0.25% SDS) for 1 hr at 65° before adding labeled probe. After an overnight incubation, filters were washed three times for 30 min each in 0.2× SET, 0.1% SDS, 0.1% sodium pyrophosphate and 25 mM phosphate buffer at either a low stringency temperature (60°) or a high stringency temperature (71–74°) as indicated in the results.

Sequence determination: Restriction fragments were subcloned into pUC119 or 118 and exonuclease III was used to create a nested set of deletions as described by HENIKOFF (1987). The resulting plasmids were transformed into either *E. coli* strain JM101 to produce DNA for single-stranded sequencing, or DH5α to produce DNA for double-stranded sequencing using standard protocols (SANGER, NICKLEN and COULSON 1977; SAMBROOK, FRITSCH and MANIATIS 1989). Sequencing reactions were performed using the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemicals, Cleveland, OH). DNA sequence was determined from both strands of all regions and analyzed using the University of Wisconsin GCG sequence analysis software package version 6.2 Copyright© 1989 John Devereaux (DEVEREAUX, HAEBERLI and SMITHIES 1984). The nucleotide sequence data of the *51B* gene reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number L04795.

Bal 31 digestion of paramecium DNA: 5 μg of wild-type stock 51 DNA, d12 (A^-) and d141 (A^-, B^-) were digested with 1 unit of Bal 31 nuclease (New England Biolabs) in a buffer containing 200 mM NaCl, 20 mM Tris-HCl (pH 8.0), 12 mM $MgCl_2$, 12 mM $CaCl_2$ and 1 mM EDTA at 30°. After either 1, 3, 5 or 10 min, EDTA was added at a concentration of 20 mM to stop the reaction and samples were placed on ice until a phenol:chloroform extraction could be performed. After ethanol precipitation, samples were digested with *Eco*RI and Southern-blotted using standard techniques (MANIATIS, FRITSCH and SAMBROOK 1982).

Genetic crosses: Mating and induction of autogamy were carried out as described by SONNEBORN (1950). All laboratory stocks used were homozygous clones due to recurring autogamy. A cross between two paramecia from two different stocks yields heterozygous F_1 exconjugant clones with identical micronuclear genotypes. F_2s are obtained by inducing autogamy in the F_1 clones. For each gene locus, half of the resulting F_2 lines are homozygous for the allele found in one parent and the other half are homozygous for the allele found in the other parent. Hence, a normal Mendelian mutation would segregate with a 1:1 ratio in the F_2 generation. Both the Mendelian marker *twisty* (*tw*) (SONNEBORN 1975), and the A serotype were used to distinguish the two parents and to indicate proper exchange of nuclei. Expression of the A serotype can be used in this manner due to the stability of its expression. Wild-type parents were expressing the A serotype at the time of mating and F_1's from the wild-type side of the cross continued to express the A surface protein.

RESULTS

Isolation and identification of B type surface antigen clones: Southern analysis of genomic digests from wild-type, d12 (A^- mutant) and d12.141 (A^-, B^- double mutant) cell lines was used to determine that a 9-kb *Eco*RI restriction fragment, present in A^- cells but not in A^-, B^- cells, hybridized with the *A* surface protein gene under low stringency conditions. These data and additional unpublished information (L. B. PREER and J. R. PREER, Indiana University) led to the conclusion that the 9-kb *Eco*RI fragment encoded a portion of the *B* surface protein gene. To clone this 9-kb *Eco*RI fragment, a genomic library was constructed in the vector λgtWES.λB using *Eco*RI digested DNA from d12, the A^- mutant. Because this library does not contain the *A* gene, it could be screened under low stringency conditions using pSA8.8R (a large portion of the A gene) as a probe. The first *B* gene clone, λSB-1, consisted of the 9-kb *Eco*RI fragment, which contained 4 kb of 5′ upstream sequence and 5 kb of the coding sequence of the *B* gene. To clone the entire *B* gene, this 9-kb insert was subcloned into pUC119 (this clone is hereafter referred to as pSB9.0R) and used as a probe to screen a wild-type *P. tetraurelia* genomic library (see MATERIALS AND METHODS). A full length clone, λSB-2 was obtained. Figure 1 shows the result of hybridizing λSB-1 to both total and poly(A)$^+$ RNA from B serotype cells and total RNA from A serotype cells. A strong signal was obtained from B RNA but no signal from the A RNA was obtained under these conditions.

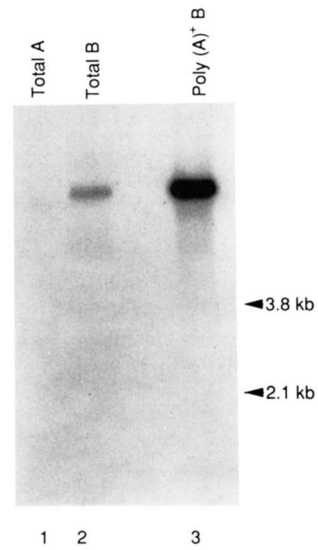

FIGURE 1.—Northern blot hybridization analysis of the cloned *B* gene. RNA from log growth cells was electrophoresed on a 1% formaldehyde gel, blotted to nitrocellulose, and then probed with the insert DNA from λSB-1, which had been subcloned into the plasmid pUC119. Lane 1, 10 μg of total RNA from cells expressing surface protein A. Lane 2, 10 μg of total RNA from cells expressing surface protein B. Lane 3, 2 μg of poly(A)+ RNA from B-expressing cells.

The *B* gene signal was greatly enhanced in the poly(A)$^+$ RNA lane, as expected for a variable surface protein mRNA (PREER *et al.* 1981).

Transcription maps of λSB-1 and λSB-2: Restriction maps were constructed for the two genomic clones (Figure 2). The size of the paramecium DNA insert in λSB-2 is approximately 17 kb. The transcribed regions were mapped by hybridizing ^{32}P-end-labeled poly(A)$^+$ RNA from B serotype cells to Southern blots of restriction fragments of the clones. The orientation of the transcription unit was determined by sequencing the ends of the transcribed regions.

The 5′ transcription start site was determined by primer extension analysis using a synthetic oligonucleotide primer. The primer, chosen approximately 50 nucleotides from the putative methionine start codon, was ^{32}P-end-labeled and hybridized to poly(A)$^+$ RNA from B serotype cells. The primer was then extended using reverse transcriptase. This produced two products that terminate at adenine nucleotides, 9 and 10 bases upstream from the ATG translation start codon (see Figure 3). A second primer, approximately 100 nucleotides from this start codon, was also extended and resulted in termination at these two nucleotides (data not shown). Our results using two different primers for extension of the *A* gene transcript indicate two products terminating at adenine residues 8 and 9 nucleotides upstream of the ATG (see Figure 3).

The 3′ end of the *B* gene transcript was located by S1 protection analysis (BERK and SHARP 1977). The 3′-1.6-kb *Pst*I fragment (see Figure 2) was 3′-end-labeled, hybridized to poly(A)$^+$ RNA from serotype B cells and then treated with various amounts of S1

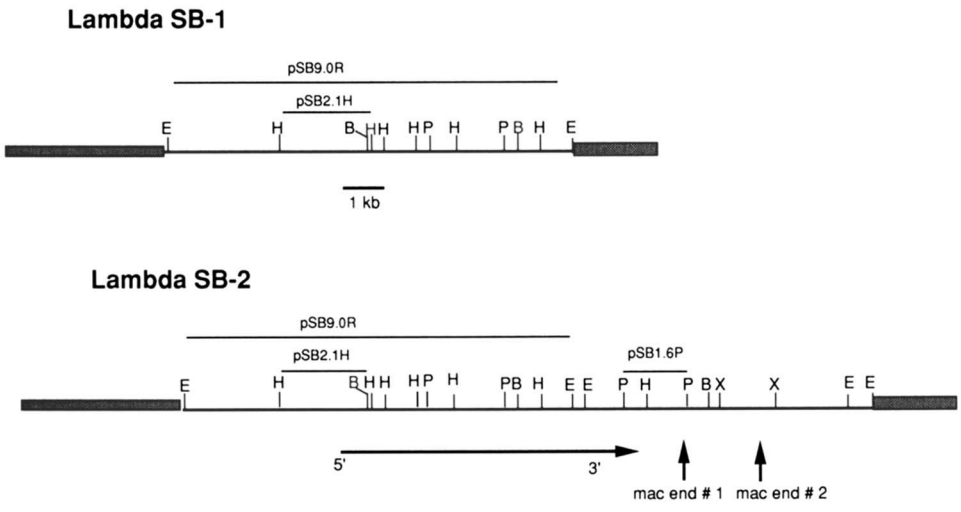

FIGURE 2.—Maps of the two *B* gene clones. The line with an arrow indicates the length of the transcript and the direction of transcription. Macronuclear chromosomal ends as determined by Bal 31 sensitivity (see text) are indicated by the arrows. Boxed regions represent phage arms (see MATERIALS AND METHODS). Selected restriction sites are indicated (B, *Bgl II*, E, *Eco*RI, H, *Hin*dIII, P, *Pst*I, and X, *Xba*I). pSB9.0R is a 9-kb *Eco*RI fragment from the first *B* gene clone λSB-1, which was subcloned into the plasmid pUC119 and used as a probe to screen the wild type genomic library. pSB 2.1H is a 2.1 *Hin*dIII fragment of the *B* gene clone λSB-1, which was subcloned into the plasmid pUC119 and used as a Southern blot probe during the genetic analysis. pSB 1.6P is a 1.6 kb *Pst*I fragment of the *B* gene clone λSB-2, which was subcloned into the plasmid pUC119 and used as a probe in S1 mapping and Bal 31 experiments.

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          -100
51A  NAACTTATTT AAACAAAATG AGGTAATTGA AAAAAAAAAA AAAATTAACA
51B  AACAATTACT AAAAACTGAT GTTTAATTAA GTTTTGTAAA AATTAAATTT

          -50
                                          **
51A  AACTAACTAT TTAAAAATTC ATTCGATTCA AATTTAATTT TAATACTTTA ATG
                                          **
51B  TATTCATAAT TTAGCTTGAT TAGAATGTAA ACCTAAAGTT AATACTTTTA ATG
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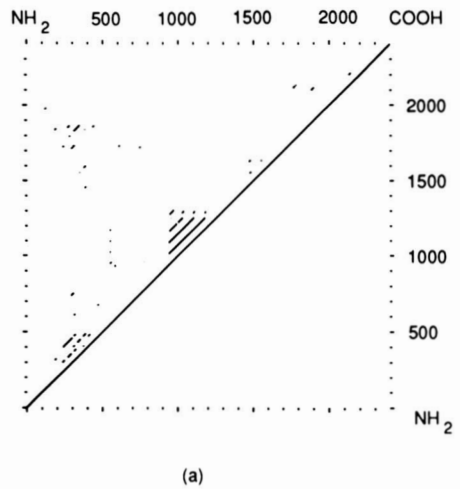
FIGURE 3.—The 5′ upstream regions of the *51A* and *51B* genes. Conserved sequences are underlined. The *51*A sequence and designation of conserved regions are taken from PREER *et al.* (1987). Transcription start sites as determined by primer extension are indicated by an asterisk (*).

nuclease. A 460-nucleotide band was protected from digestion, suggesting that the end of transcription lies 460 nucleotides from the *Pst*I site (data not shown). This would place the end of the transcript approximately 70 nucleotides from the TGA stop codon. This is consistent with previous results showing the 3′ end of the *A* gene is 30 nucleotides from the TGA stop codon and the *G* and *H* genes terminate roughly 55 nucleotides downstream of the TGA stop codon (PREER *et al.* 1987; GODISKA 1987; PRAT *et al.* 1986).

DNA sequence of the B surface protein gene: The complete nucleotide sequence for the coding region of the *B* type surface antigen gene was determined. It has an open reading frame of 7,182 nucleotides that encodes a primary translation product of 2,394 amino acids. The calculated molecular weight for the B polypeptide is 246,246 daltons, which is within 5% of the experimental value of 259,000 (REISNER *et al.* 1969). The slightly smaller calculated value would obviously not include the carbohydrate portion of the glycoprotein. The gene has one continuous open reading frame that is not interrupted by stop codons. The upstream region of the *B* gene contains two sequences that are very similar to the *A* gene: a region just ahead of the ATG start codon, and a long AT-rich stretch located at approximately −50 (see Figure 3). These conserved sequences have previously been identified in the *51C*, *51H* and *156G* surface antigen genes (PREER *et al.* 1987).

Like all the surface protein genes from paramecium so far examined, the *B* gene encodes an extremely cysteine, serine and threonine-rich protein, being 11.1, 9.9 and 16.2 mole percent, respectively. Regular spacing of cysteine residues defines a periodic structure of the protein that appears to be a general feature of paramecium surface proteins (PRAT *et al.* 1986; PRAT 1990; NIELSEN, YOU and FORNEY 1991). A dot matrix analysis (Figure 4) reveals that as in the *A* surface protein gene from *P. tetraurelia* and the *G* alleles from *P. primaurelia*, the *B* gene also contains tandem repeats in the central coding region. There are three nearly perfect tandem repeats of 219 nucleotides in the center of the *B* gene. This compares with 210-bp repeats in the *A* gene and 222-bp tandem repeats in the *G* gene. The central 600 amino acids (800–1400) of B are only 59% identical to A, whereas the amino and carboxyl termini show identity of 85% and 88%, respectively. Both A and B belong to the same antigenic family. In comparison, B and C are immunologically unrelated and show 59% and 62% identity for the amino and carboxy terminal regions, and only 43% identity in the central region.

Macronuclear location of the B surface protein gene: In the macronucleus, both the *A* gene of *P. tetraurelia* stock 51 and the *G* gene of *P. primaurelia* are located near macronuclear telomeres (FORNEY and BLACKBURN 1988; MEYER, CARON and BAROIN 1985).



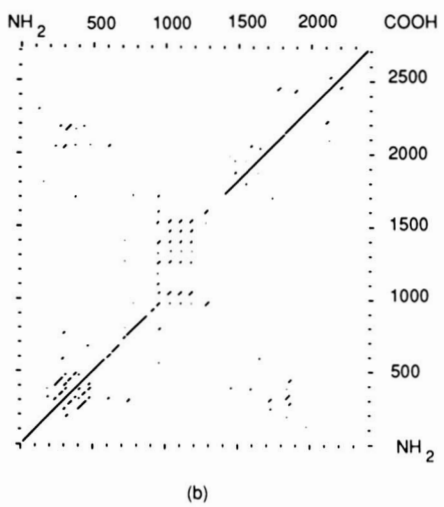


FIGURE 4.—Analysis of the 51B deduced amino acid sequence for internal repeats and comparison with 51A. Dot matrix analysis was performed on the deduced amino acid sequence of 51B and 51A. A window of 30 amino acids and a stringency of 25 matches were used for both panels. (a) 51B *vs.* 51B; (b) 51B *vs.* 51A. The deduced amino acid sequence for 51A was previously reported (NIELSEN, YOU and FORNEY 1991). The nucleotide sequence data reported in this paper will appear in EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number L04795 (*51B*).

To determine whether the *B* surface protein gene is also located near a telomere, we analyzed the sensitivity of sequences located distal to the the 3′ end of the cloned *B* gene to Bal 31 nuclease digestion. Samples of macronuclear DNA were submitted to Bal 31 digestion for increasing periods of time and then digested with the restriction enzyme *Eco*RI. The genomic digests were electrophoresed on an agarose gel, blotted to nitrocellulose and probed with the 1.6-kb *Pst*I fragment from the 3′ end of the *B* gene. The blots were washed stringently (72°, 0.2× SET) to reduce signals from *B*-related sequences.

The analysis of macronuclear DNA in ciliates can be complicated due to alternative DNA rearrangements that occur during macronuclear development (BLACKBURN and KARRER 1986; YAO 1989). In paramecium, the telomeric regions apparently have a large number of heterogeneous restriction fragments as a result of alternative DNA rearrangements and/or breakage sites (FORNEY and BLACKBURN 1988; CARON 1992). The Bal 31 digestion probed with the *B* gene shown in Figure 5 reveals that a 4.5-kb band is progressively shortened by Bal 31. A second band approximately 2.5 kb in size is also Bal 31 sensitive. Both of these bands are present in d12 (A^-) DNA but not in d12.141(A^-, B^-) DNA. A third approximately 7.0-kb *EcoR1* band present in wild-type and d12 (A^-) represents a longer version of a macronuclear chromosome on which the *B* gene resides. This longer version is the one present in the clone λSB-2 (see *B* gene map, Figure 2). The preferential Bal 31 digestion of these bands indicates that they are telomeric restriction fragments. To demonstrate that all of these bands are forms of the *B* locus, seven F_2 progeny of a cross between d12.141 (A^-, B^-) and wild-type cells were examined by Southern analysis for the presence or absence of these bands. The three representative B^+ F_2 cell lines we examined each contained all three bands, while the four B^- F_2 progeny were lacking all three bands (data not shown). Hence, the three bands cosegregate with the B expression phenotype, and therefore must represent three alternate forms of the macronuclear chromosome that are the result of variable DNA processing events that occur during formation of the macronucleus. The variable nature of macronuclear fragments is evident from the wild-type stock 51 DNA that was used as a control (Figure 5, lane 1). It did not contain the 4.5-kb *B* gene band, but appeared to have a greater proportion of the 2.5-

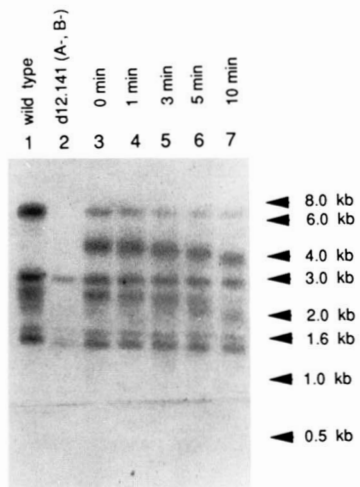


FIGURE 5.—Bal 31 sensitivity of *B* gene bands. d12 (A^-) DNA was treated for either 0, 1, 3, 5 or 10 min (lanes 3–7) with the nuclease Bal 31 and then digested with *Eco*RI. The DNA was then electrophoresed, blotted and probed with the 3′ 1.6-kb *Pst*I fragment of the *B* gene (see Figure 2). A 4.5-kb band is sensitive to Bal 31 digestion. A second 2.5-kb band is Bal 31 sensitive. Lane 1, *Eco*RI digested wild-type stock 51 DNA. Lane 2, *Eco*RI digested d12.141 DNA.

kb and 7-kb bands. Although the 4.5-kb band is missing in the macronuclear DNA from this particular DNA preparation, we have found this band in other wild-type preparations of DNA.

The size heterogeneity of the Bal 31 sensitive bands is characteristic of fragments derived from the ends of macronuclear chromosomes and is due to the variability in the number and precise location of G_4T_2 and G_3T_3 terminal repeats in telomeric regions (FORNEY and BLACKBURN 1988). The size of the Bal 31 sensitive bands reflect the distance from the *Eco*RI site in the *B* gene to the end of a macronuclear chromosome. Thus, the macronuclear telomere downstream of the *B* gene can be found in at least three locations. Two of the sites are approximately 1 and 3 kb downstream from the *B* gene, the other is not known, but must be more than 6 kb away from the 3′ end of the *B* gene (see *B* gene map, Figure 2).

To determine the size of the specific macronuclear fragments that contain the *B* gene, undigested, total cellular DNA was electrophoresed on a pulsed field gel, blotted to nitrocellulose and hybridized to pSB9.0R, a plasmid containing a large portion of the cloned *B* gene. Two discernable bands, approximately 245 kb and 275 kb in size, were visible. Both of these bands were missing in d12.141 DNA (data not shown). The relationship between these two macronuclear chromosomes and the alternative locations of the macronuclear telomeres is not known.

Genetic analysis of A^-, B^- mutants

F_1 analysis: Because the four A^-, B^- mutants were derived from the A^- mutant d12, it is known that they contain complete deletions of the *A* gene. Southern analysis using probes from both the 5′ and 3′ regions of the cloned *B* gene was used to determine that all four of the A^-, B^- mutants contained complete macronuclear deletions of the *B* gene as well (data not shown). To determine if the inheritance of the B^- trait in any of the mutant cell lines is affected by the cytoplasm of the cell like the A^- trait in d48, we crossed all four mutants to stock 51, wild-type cells and analyzed the genomic DNA obtained from the F_1 generation. If the inheritance of any of the *B* gene mutations is similar to that of d48, then the F_1 macronucleus from the wild-type side of the cross would contain normal amounts of the *B* gene and the mutant side F_1 macronucleus would have no detectable *B* gene (EPSTEIN and FORNEY 1984). Figure 6 shows Southern blots of *Hin*dIII digested genomic DNA from F_1 progeny from a cross between wild-type and d12.141 cells (lanes 4 and 5), and a cross between wild-type and d12.144 cells (lanes 6 and 7). The progeny are descendents of either the wild-type cytoplasmic parent (lanes 4 and 6), or the A^-, B^- mutant cytoplasmic parent (lanes 5 and 7). The DNA was probed with pSB2.1H, a 2.1-kb *Hin*dIII subclone of the *B* gene (see Figure 2) and pSA1.4H, a 1.4-kb *Hin*dIII subclone of the *A* gene. Since all four of the mutants are derived from the Mendelian d12 mutant, and previous studies have shown that F_1 exconjugants from d12 contain equal amounts of the *A* gene on each side of the cross, the A probe was used as a control. Wild-type, d12 (A^-) and the parental mutant (A^-,B^-) were included as controls on the Southern blots and high stringency conditions were used to eliminate any hybridization between the *A* and *B* genes. As shown in Figure 6, both the *A* and the *B* gene appear in F_1 exconjugants regardless of the cytoplasmic parent, indicating that the inheritance pattern of these mutants is not like that of d48. The analysis of F_1 exconjugants from the other two mutants, d12.59 and d12.138, produced identical results (data not shown).

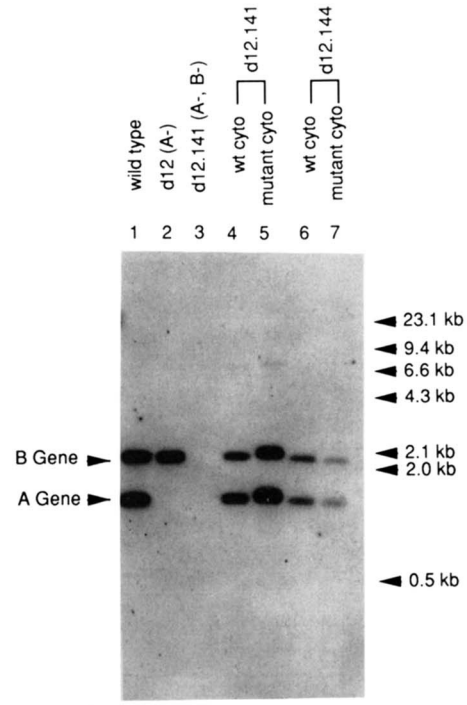


FIGURE 6.—Genomic Southern analysis of F_1 heterozygotes. DNA was obtained from both F_1 exconjugants in a cross between wild-type and d12.141 cells (lanes 4 and 5) and in a cross between wild-type and d12.144 cells (lanes 6 and 7). DNA was digested with *Hin*dIII, electrophoresed, blotted and then simultaneously probed with pSA1.4H and pSB2.1H, two plasmids that contain *Hin*dIII fragments from the *A* gene and *B* gene, respectively. The *A* and the *B* gene are present in both exconjugants (differences in signal intensities may be accounted for by differences in the amount of DNA loaded in each lane). F_1 cell lines descended from wild-type parental cytoplasm, lanes 4 and 6. F_1 cell lines descended from mutant parental cytoplasm, lanes 5 and 7. Lane 1, wild-type stock 51 DNA. Lane 2, d12 DNA. Lane 3, d12.141 (parental mutant) DNA.

F_2 analysis: An F_2 generation was produced by autogamy for three of the mutants, d12.138, d12.141 and d12.144. Because reliable induction of serotype B expression is more time consuming than serotype A induction, Southern analysis was used to score the F_2 progeny. Total DNA was isolated from F_2 cell lines, digested with *Hin*dIII and then probed with the same fragments used to analyze the F_1 individuals, pSA1.4H

and pSB2.1H. An F_2 cell line was considered A^+ if it contained the 1.4-kb *Hin*dIII fragment of the *A* gene and B^+ if it contained the 2.1-kb *Hin*dIII fragment of the *B* gene on a Southern blot. Representative individuals of each genotypic class were chosen from each cross and grown under conditions to induce serotype A expression (34°) or B expression (19° in wheat grass media containing 10 μg/ml $ZnCl_2$), and then scored for A or B using the standard antisera assay. In every case examined, the A and B serotype expression was correlated with the presence of the A and B *Hin*dIII fragments, respectively, on a genomic Southern. The unusual results of the d12.138 and d12.144 crosses forced us to use *twisty* (*tw*) as well as the *A* gene as a genetic marker.

Crosses of d12.141 to wild-type cells resulted in acceptable 1:1 ratios in the F_2 generation for both the A^- trait (as expected) and the B^- trait (Table 1). In contrast to the straightforward Mendelian inheritance of d12.141, the segregation of traits among F_2 progeny from crosses of d12.138 and d12.144 to wild type was more complex. In both crosses all four classes of progeny were obtained, and as expected from the F_1 analysis, no effect of the parental cytoplasm on inheritance was observed (Table 1). Clearly, these two mutant lines do not show the cytoplasmic inheritance typical of d48. Nevertheless, in both crosses the ratios of A^+ to A^- cells and B^+ to B^- cells had unacceptable chi-square values for the Mendelian null hypothesis: these values range from 5.56 and 14.0 for the segregation of the A^+ and B^+ traits, respectively, in d12.144, to 10.6 and 8.5 for the same traits in the d12.138 to wild-type cross. Examination of the macronuclear genotypes in Table 1 shows that both crosses resulted in an underrepresentation of the A^- and B^- traits. This occurred despite an acceptable chi-square value for the genetic marker *twisty* (a cell body deformation) in both crosses (0.942 for the d12.144 cross and 0.735 for the d12.138 cross, see Table 1). The explanation for these results is not obvious since the A^- allele of d12 is known to segregate in a Mendelian fashion when crossed to wild-type cells (RUDMAN *et al.* 1991). However, the mortality rate of the F_2 cell lines was quite high for all crosses and selective death of particular genotypic classes cannot be ruled out.

Unusual F_2 progeny: Examination of the genomic Southerns of F_2 progeny from the d12.144 to wild-type cross revealed that 10 of 50 cell lines appeared to have abnormally low copy numbers of the *A* and/or *B* gene. Six F_2s had less than the expected signal for *A*, two had less *B* and two had reduced signals for both *A* and *B*. Since autogamy results in two caryonides (cells with independently formed macronuclei), we initially suspected that these unusual cell lines might contain a mixed population, such that most cells had complete deletions of the *A* or *B* gene and a few cells had normal copy numbers. To test this possibility, single cells from six F_2 lines were isolated to initiate subcultures for Southern blot analysis. All of the six F_2 lines examined produced subcultures with reduced signals for the same genes as the original F_2 lines. For five of the lines the results were identical to the original cultures, and in one there appeared to be some variation in the signal intensity of the *A* gene among the subcultures. Figure 7 shows, as an example, a Southern hybridization that contains two subcultures of an F_2 line with less than wild-type *A* gene copy number (lanes 6 and 7) and two subcultures of another F_2 line with a lower copy number of both the *A* and *B* genes (lanes 4 and 5). To normalize the data for the amount of DNA loaded in each lane, the blot was reprobed with a fragment of the *C* gene (Figure 7, lower panel). Digests with additional restriction en-

TABLE 1

Inheritance of A^-, B^-, mutant traits

Cross	Progeny of 51 exconjugant	Progeny of mutant exconjugant	Total progeny
d12.141 × 51 (wt)	$A^+, B^+ = 9$	$A^+, B^+ = 6$	$A^+, B^+ = 15$
Sum of 3 pairs	$A^-, B^+ = 4$	$A^-, B^+ = 6$	$A^-, B^+ = 10$
	$A^+, B^- = 1$	$A^+, B^- = 4$	$A^+, B^- = 5$
	$A^-, B^- = 5$	$A^-, B^- = 5$	$A^-, B^- = 10$
	$A^+:A^- = 20:20$ [$\chi 2 = 0.025$, $P > 0.80$]		Sum = 40
	$B^+:B^- = 25:15$ [$\chi 2 = 2.02$, $p > 0.20$]		
d12.144 × 51 (wt)	$A^+, B^+ = 11$	$A^+, B^+ = 21$	$A^+, B^+ = 32$
1 pair	$A^-, B^+ = 6$	$A^-, B^+ = 2$	$A^-, B^+ = 8$
	$A^+, B^- = 2$	$A^+, B^- = 1$	$A^+, B^- = 3$
	$A^-, B^- = 4$	$A^-, B^- = 5$	$A^-, B^- = 9$
	$tw^+ = 13$	$tw^+ = 17$	$tw^+ = 30$
	$tw = 10$	$tw = 12$	$tw = 22$
	$A^+:A^- = 35:17$ [$\chi 2 = 5.56$, $P < 0.05$]		Sum = 52
	$B^+:B^- = 40:12$ [$\chi 2 = 14.0$, $P < 0.01$]		
	$tw^+:tw = 30:22$ [$\chi 2 = 0.942$, $P > 0.20$]		
d12.138 × 51 (wt)	$A^+, B^+ = 13$	$A^+, B^+ = 8$	$A^+, B^+ = 21$
1 pair	$A^-, B^+ = 4$	$A^-, B^+ = 1$	$A^-, B^+ = 5$
	$A^+, B^- = 3$	$A^+, B^- = 3$	$A^+, B^- = 6$
	$A^-, B^- = 0$	$A^-, B^- = 2$	$A^-, B^- = 2$
	$tw^+ = 6$	$tw^+ = 8$	$tw^+ = 14$
	$tw = 14$	$tw = 6$	$tw = 20$
$A^+:A^- = 27:7$ [$\chi 2 = 10.6$, $P < 0.01$]			Sum = 34
$B^+:B^- = 26:8$ [$\chi 2 = 8.50$, $P < 0.01$]			
	$tw^+:tw = 14:20$ [$\chi 2 = 0.735$, $P > 0.20$]		

DNA was obtained from all F_2 progeny, digested with *Hin*dIII, electrophoresed, blotted and then simultaneously probed with pSA1.4H, and pSB2.1H, two plasmids which contain *Hin*dIII fragments from the *A* gene and *B* gene, respectively. F_2 progeny were considered A^+ if they contained the 1.4-kb *Hin*dIII fragment of the *A* gene, and B^+ if they contained the 2.1-kb *Hin*dIII fragment of the *B* gene on a Southern blot, even if the signal was reduced in intensity compared to wild type. The null hypothesis of 1:1 segregation of the F_2 progeny was tested by chi-square.

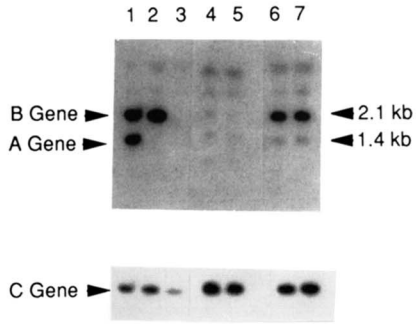


FIGURE 7.—Analysis of unusual F_2 cell lines from a cross between wild-type and d12.144 cells. Single cell subcultures analyzed were from two different F_2 cell lines that originally showed reduced hybridization signals for either the *A* gene (lanes 6 and 7) or both the *A* and *B* genes (lanes 4 and 5). DNA was isolated from each subculture, digested with *Hin*dIII and analyzed by Southern hybridization. The blot was probed with a nick-translated mixture of pSA1.4H and pSB2.1H, which contain *Hin*dIII fragments from the *A* and *B* genes, respectively. Lane 1, wild-type stock 51 DNA. Lane 2, d12 DNA. Lane 3, d12.144 DNA. Lower panel, same blot stripped and reprobed with a portion of the *C* gene to normalize the amount of DNA loaded in each lane.

zymes were performed to ensure that these bands represent the authentic *A* gene as opposed to related cross-hybridizing sequences (data not shown). The cell line shown in Figure 7 that had a reduced copy number of both the *A* and *B* genes was taken through autogamy and the Southern analysis was repeated. The results demonstrated that although there was some variation among individual cell lines, all of the new macronuclei had lower copy numbers of the *A* and *B* genes as did the original line (data not shown).

DISCUSSION

The *51B* surface protein gene: We have cloned the gene encoding the B surface protein from stock 51 of *P. tetraurelia*. The *B* gene, like the other variable surface protein genes examined in paramecium to date, does not contain introns. Primer extension and S1 analysis reveal that both the 5′ and 3′ transcribed, nontranslated regions are relatively short. The transcription start site is located only 9 or 10 nucleotides upstream of the ATG start codon, while the 3′ transcribed, nontranslated region is about 70 nucleotides in length. Our primer extension results for the 5′ end of the *A* gene indicates two transcriptional start sites at two adenine residues 8 and 9 nucleotides upstream of the ATG. These two A residues are within a conserved sequence found in the upstream regions of all paramecium variable surface protein genes, and the one nucleotide difference between the transcription start of *A* and *B* is correlated with an additional T residue 5 nucleotides upstream from the ATG codon in *B*. Thus, our data indicate similar transcription start sites for the *A* and *B* genes. Previous primer extension results placed the transcription start for the *A* gene at 7 nucleotides upstream of the ATG (JESSOP-MURRAY *et al.* 1991).

The 5′ upstream region of the *51B* gene contains two conserved sequences previously identified in the *156G*, *51A*, *51C* and *51H* genes: a conserved region immediately before the start of translation and a long AT-rich stretch at −70 to −40 (PREER *et al.* 1987). The 10-nucleotide sequence AATTAATGAT found between positions −160 and −120 in *156G*, *51A*, *51C* and *51H* cannot be identified in the *51B* upstream region.

Like all paramecium variable surface proteins, B has a remarkably high cysteine, threonine and serine content. In all cases the cysteine residues define a periodic structure that consists of units roughly 70 amino acids in length. The deduced amino acid sequence of *51B* contains three nearly perfect tandem repeats in the central region of the molecule. Similar internal tandem repeats have been found in the *51A*, *156G* and *168G* proteins, but not in *51C* (NIELSEN, YOU and FORNEY 1991; MEYER, CARON and BAROIN 1985; PRAT 1990). Interestingly, the proteins that have tandem repeats (A, B and G) are most closely related to each other, and are encoded by telomeric genes. In contrast, the C protein, which is the most divergent, is encoded by a gene further from the telomere (J. FORNEY, unpublished results) and does not have tandem repeats. As earlier studies have indicated, the most conserved regions of the surface proteins are the amino and carboxyl terminal regions (PRAT 1990; NIELSEN, YOU and FORNEY 1991). In contrast, the central 500–700 amino acid regions are the most divergent. The central 600 amino acids of the B surface protein show only 59% identity to the A protein, even though these two proteins are from the same antigenic family. Hence, the central regions are not well conserved among even the most closely related family members.

There is increasing evidence that paramecium surface protein genes are preferentially located near macronuclear telomeres. The *G* surface protein gene in *P. primaurelia* is located at about 5 kb from a macronuclear telomere (MEYER, CARON and BAROIN 1985). As a result of variable DNA processing events during macronuclear development, the *A* gene is located at either 8, 13 or 26 kb upstream from a macronuclear telomere (FORNEY and BLACKBURN 1988). In this report we demonstrate that the *B* surface protein gene is also located near a macronuclear telomere, with the most proximal telomere just 1 kb from the 3′ end of the gene. It is not yet known whether a telomeric location has any functional significance for paramecium variable surface protein gene expression, but the striking similarity to trypanosome variable surface protein genes is intriguing. In parasitic trypanosomes, surface protein genes can only be transcribed if they are located in special telomeric expression sites (reviewed in CROSS 1990). Since the number of expression sites per genome is well below the number of

surface protein genes, these genes must be moved in and out of the telomeric sites. Although there is no evidence for such DNA rearrangements of the serotype genes in paramecium, it is possible that a permanent telomeric location is important for proper expression (as suggested by PREER 1986).

Genetic analysis of the A^-, B^- mutants: The genetic analysis of macronuclear deletions of the *A* gene has revealed two classes of inheritance: Mendelian mutations that segregate in a 1:1 ratio in the F_2 generation, and non-Mendelian mutations that result in mutant progeny from the mutant parental exconjugant and wild-type progeny from the wild-type parent (EPSTEIN and FORNEY 1984; RUDMAN *et al.* 1991). The non-Mendelian mutant d48 has a normal copy of the *A* gene in its micronucleus, but this copy is not incorporated into the macronucleus. In contrast, the d12 (A^-) mutant must have some alteration in its micronuclear DNA. The Mendelian mutant d12 can be distinguished from d48 experimentally by analysis of F_1 exconjugants. d12/+ heterozygous cell lines express A and contain copies of the *A* gene in their macronuclei, but d48/+ heterozygous F_1 lines generally do not have copies of the *A* gene in the macronucleus derived from the d48 parent. All four of the A^-, B^- macronuclear deletion mutants when crossed to wild-type cells contained equal amounts of the *A* and *B* genes in F_1 cell lines from both exconjugant lines. These results clearly indicate a d12-like pattern of inheritance for the *B* gene. In addition, no effect of the parental cytoplasm was detected in determining the macronuclear genotype of F_2 progeny from d12.141, d12.144 and d12.138 crosses to wild-type cells. The d12.141 cell line displayed a typical 1:1 Mendelian ratio for the *A* and *B* gene traits in the F_2 generation; thus, it represents an example of a Mendelian deletion of the *B* gene. The d12.144 and d12.138 cell lines when crossed to wild type produced an excess of A^+ and B^+ cells in the F_2 generation. This cannot merely be the result of linkage since there is no correspondingly large number of A^-, B^- progeny. The mortality rate of the F_2 cell lines was quite high for all crosses. The A^-, B^- cell lines are the result of two successive rounds of mutagenesis. Although these homozygous cell lines cannot contain recessive lethal mutations, accumulated chromosomal defects could result in new lethal combinations after a genetic cross. Nevertheless, the ratio of B^+ to B^- cells is similar to the ratio of A^+ to A^- cells and since d12 is known to segregate in a Mendelian fashion, it is likely that the B^- mutations would also show Mendelian inheritance if backcrossed sufficiently to eliminate any mortality effects.

Remarkably, the d12.144 cross resulted in several unusual F_2 lines that had less than normal hybridization signals on Southern blots for either the *A* or *B* gene. These lines were identified because Southern blot analysis was performed on all F_2 lines and would have been overlooked if only phenotypic analysis was performed. In fact, phenotypic scoring of the d12.144 cross would have resulted in an acceptable Mendelian segregation ratio for A expression because the low copy number F_2 lines cannot express A. We have shown that these lines stably maintain lower copy numbers of the *A* and *B* genes even after formation of a new macronucleus; consequently, they must contain copies of the *A* and *B* genes in their micronuclei. The presence of complete micronuclear genes yet defective macronuclei suggests these cell lines may be examples of non-Mendelian mutants that have been generated from the genetic cross. This can be confirmed by a genetic analysis or permanent rescue of the cell lines by transformation with the *A* or *B* genes, as previously described for d48 (YOU *et al.* 1991; JESSOP-MURRAY *et al.* 1991). If these cell lines are examples of non-Mendelian mutants they will be useful for studies on the specificity of non-Mendelian effects. The mechanism by which these unusual F_2 lines or the d48 cell line arose is not understood. Presumably an error is made during the formation of a new macronucleus that results in low copy number of the *A* gene. Once made, the error is set and cannot be corrected despite subsequent nuclear reorganizations unless additional copies of the *A* gene are included in the old macronucleus. It is possible that this effect is specific for telomeric genes in paramecium, but currently little direct evidence is available to support this idea. From a practical viewpoint, the isolation of these unusual F_2 lines underscores the possible complications of phenotypic genetic analysis in paramecium. Although we do not expect these effects to occur for point mutations, the genotypic defect for most mutations in paramecium is not known, and unusual macronuclear effects could complicate the analysis if the mutation results from a deletion.

The cloning of the *B* surface protein gene and characterization of the *B* gene deletion mutant cell lines will allow more sophisticated studies of surface protein gene expression and non-Mendelian inheritance. Examples include the expression of chimeric *A*/*B* surface protein genes without interference by the endogenous native genes, and construction of a non-Mendelian B^- mutant by transfer of a wild-type micronucleus into the d12.141 cell line. The latter technique has already been used to create a d48 cell line by transferring a wild-type micronucleus into an amicronucleate d12 cell (KOBAYASHI and KOIZUMI 1990; Rudman *et al.* 1991). If the same procedure can be used to create a B^- non-Mendelian mutant, it will demonstrate the generality of this mechanism, and the resulting mutant will be a useful tool to study the specificity of the sequences that control the incorporation of the *A* and *B* genes into the developing macronucleus.

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