

Analysis of *Paramecium tetraurelia* A-51 Surface Antigen Gene Mutants Reveals Positive-Feedback Mechanisms for Maintenance of Expression and Temperature-Induced Activation

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In *Paramecium tetraurelia*, variable surface antigen loci show mutually exclusive expression which is controlled primarily at the transcriptional level. Clonally stable expression of a single antigen has attracted models involving self-regulation by their gene products. However, direct demonstration of self-feedback at the molecular level has been complicated due to the inability to separate the functional gene from its product as well as copy number effects associated with injected extrachromosomal DNA in the polygenomic somatic nucleus. In this study, we exploited several germ line termination and frameshift mutations in the A-51 surface antigen gene to analyze variable surface antigen expression. These mutant alleles have the same copy number as the wild-type allele and therefore eliminate possible copy number effects. The mutant alleles were not transcribed at 27°C, consistent with positive-feedback models for gene expression. However, further analysis showed that high temperatures (34°C) induced transcription of the mutant A genes even in the presence of a different antigen on the cell surface. Thus, transcription was temperature dependent. Unlike wild-type cells, transcription of the mutant A genes at high temperatures was not maintained after temperature shift back to 27°C in homozygous mutant cells. Importantly, transcription of the mutant allele was maintained at 27°C in heterozygous cells with one copy of the wild-type allele. These results indicate that expression of the wild-type gene is required to stabilize its own transcriptional state at 27°C.

Heritable traits of eukaryotic cells are not always governed by DNA sequence information in the nucleus. Examples of heritable traits independent from DNA information include higher-order chromatin structures (18, 20), propagation of protein structures (44), and stable expression of variable surface antigens in protozoa and fungi (16, 27, 29, 31, 39).

The free-living ciliated protozoan *Paramecium tetraurelia* has a system for variable surface antigen expression (5, 6, 21, 30) that has some general similarity to parasitic protozoa. Wild-type stock 51s can express at least 11 different surface antigens also known as immobilization antigens (i-antigen) designated A51, B51, C51, and so on. Each gene is encoded by an unlinked locus, yet only one protein is stably presented on the cell surface at a given time. Thus, antigen expression is mutually exclusive. Each antigen type, called a serotype, is inherited through clonal generations and sexual generations unless its expression is replaced by another antigen (36).

The mechanism controlling mutual exclusion is still unclear. Earlier genetic, physiological, and biochemical studies on surface antigen switching (3, 10, 19, 35) have suggested that expression of a single antigen locus is maintained by self-feedback from its gene product. This attractive hypothesis has, however, not been directly demonstrated. Although switching of surface antigen expression may occur spontaneously at a low frequency, directed switching toward particular antigen types can be induced by sudden changes in environmental factors such as temperature (1, 35). Although identical temperatures

can be used to culture cells with different antigenic types, expression of A51 is favored by high temperatures (34°C) and H51 is normally expressed at low temperatures (14°C).

Paramecium surface antigen proteins are abundant 250- to 310-kDa glycoproteins that account for about 3.5% of total protein (30). The nucleotide sequences of the surface antigen genes A-51, B-51, C-51, α D-51, G-51, and H-51 have been analyzed (2, 8, 14, 28, 32). The predicted amino acid sequences show that the N-terminal one-third and C-terminal one-third are more conserved than the central region. The N termini contain a probable signal peptide to deliver the proteins to the cell surface, while the C termini are modified with a glycosylphosphatidylinositol anchor to attach the proteins to the plasma membrane (4). Like surface antigens in parasitic protozoa, a periodic spacing of cysteine residues was found in all *Paramecium* surface antigens, and at least some cysteine repeat units are necessary for A-51 gene expression (40). In addition, tandem repeats consisting of ~210 bp (or ~70 corresponding amino acids) are found in the middle of the coding regions of A-51, B-51, and G-51 (28, 33). This encodes the probable antigenic region of the protein which is exposed to the environment in vivo.

Molecular analysis has been conducted to unravel the control mechanism of mutual exclusion in *Paramecium*. Unlike other surface antigen systems such as trypanosomes (29, 31), gene rearrangement does not seem to associate with antigen switching in *Paramecium* (11, 14). In vitro nuclear run-on transcription assays revealed that expression of most *Paramecium* antigens is controlled at the transcriptional level (13). Analyses of chimeric A-51 and B-51 transgenes have shown that the 5'-upstream region of the A-51 gene is required for transcription but is not sufficient for mutual exclusion (23). The element

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responsible for mutual exclusion is at least in part located in the 5' coding region, substitution of which allowed coexpression of *A-51* with *B-51* (22). Furthermore, introduction of a frameshift mutation in this chimeric gene reduced the rate of transcription of the corresponding gene (41). These studies present evidence supporting the hypothesis that functional A51 protein is part of the feedback pathway enabling transcription of its own gene.

To analyze the positive-feedback mechanism more closely at the molecular level, in this study, we exploited several germ line termination and frameshift mutants with mutations at various positions in the *A-51* coding region. Unlike the injection of transgenes that was used in the previous studies, germ line mutants are free from problems associated with variable copy numbers of the transgene. The experiments show that the regulation of surface antigen gene expression is temperature dependent and, at 27°C, the functional gene product is required for maintenance of the transcriptional state of the corresponding gene.

MATERIALS AND METHODS

Cell lines, media, and growth conditions. *Paramecium tetraurelia* stock 51s was the parent strain for the isolation of mutants. Genetic crosses and Northern analyses used nd6 as the wild-type strain (hereafter referred to as the wild type). nd6 is homozygous for the *A-51* surface antigen allele and a recessive Mendelian mutation *nd6* that prevents trichocyst discharge. A⁻ cell lines used in this study include AIM2 (*a-aim2/a-aim2*), AIM3 (*a-aim3/a-aim3*), and AIM5 (*a-aim5/a-aim5*) (25, 26) as well as new frameshift mutants (see below). These A⁻ cell lines were backcrossed to nd6 between one and four times depending on the cell lines. Cells were cultured in a pea medium (1.25 to 2.5 g Austrian winter pea in 800 ml double-distilled water prepared in the autoclave for 20 min [42]) buffered with K-DS (4 mM sodium citrate, 2.8 mM sodium phosphate [dibasic], 1.2 mM potassium phosphate [monobasic], 1.5 mM calcium chloride, modified from Dryl's original solution [7]) supplemented with 1.25 mg/liter stigmasterol. The peas were purchased from Outsidepride.com, Inc. The medium was inoculated with *Klebsiella pneumoniae* 1 or 2 days prior to use. All cell lines were cultured by the method of Sonneborn (37) at 27°C unless otherwise noted.

Scoring for serotypes and trichocyst discharge. Expression of the antigen types was scored under a microscope at low magnification 1 hour after mixing 50 µl of cells (approximately 50 cells) and 50 µl of antisera diluted in Dryl's solution (7). Trichocyst discharge was scored by mixing 10 to 20 cells with a drop of saturated picric acid.

Isolation of mutants. Approximately 6 × 10⁵ well-fed 51s cells expressing A51 surface protein were concentrated by centrifugation into a volume of 100 ml. The cells were treated for 1 hour with an equal volume of 0.15 mg/ml nitrosoguanidine (1-methyl-3-nitro-1-nitrosoguanidine) dissolved in Dryl's solution. After mutagenesis, the cells were divided into 60 different tubes and starved to induce autogamy (self-fertilization). The resulting homozygous cells were cultured for 8 to 10 fissions at 34°C to induce A51 expression and then treated with anti-A51 serum. The surviving cells were grown for an additional 8 to 10 fissions at 34°C and again treated with anti-A51 serum. Finally, clonal lines were isolated and scored for A51 expression.

Genetic crosses. The induction of mating and autogamy was carried out by the method of Sonneborn (37). A cross between two *Paramecium* cell lines produces heterozygous F₁ exconjugant clones with identical genotypes. In some experiments, homozygous F₂ lines were obtained by inducing autogamy in the F₁ clones. A normal Mendelian mutation segregates with a 1:1 ratio in the F₂ generation by autogamy. The recessive Mendelian marker *nd6* was used as a genetic marker for the crosses.

PCR and direct sequencing for the *A* gene. Twelve primers were designed to amplify overlapping 1.5- to 3.0-kb DNA fragments that cover the entire 10.1-kb Sall-XhoI fragment of the *A* locus which contains the functional gene. The DNA fragments were amplified using proofreading DNA polymerases of Vent DNA polymerase (New England Biolabs) or EXTaq DNA polymerase (Takara Inc., Japan). PCR products were purified with UltraClean PCR clean up kit (MO BIO, Carlsbad, CA) and sent for sequencing to the Purdue Genomics DNA Sequencing Low Throughput Laboratory. The above primers plus 16 additional primers were used for sequencing the entire region at least twice. Primer se-

quences are available on request. Mutations were confirmed by sequencing independently amplified PCR products and sequence analysis of PCR products from F₂ progeny with the mutant phenotype from the cross (see Results).

Total RNA isolation. Total RNA from 50 to 100 ml of cell culture (1,000 cells/ml) was isolated as previously described (24) using the RNeasy mini kit (QIAGEN) supplemented by QIA shredder (QIAGEN) for homogenization and the RNase-free DNase set (QIAGEN) for genomic DNA elimination according to the manufacturer's instructions. The RNA samples were stored in distilled water or in ethanol at -70°C.

Nuclear run-on transcription assays. Nuclear isolation and run-on reactions were performed as previously described (13). Labeled RNA transcripts prepared from 100 µl of cytoskeletal frameworks were purified by phenol-chloroform extraction and Sephadex G50 columns. The plasmid probes (0.5 µg) used for hybridization include pSA1.4H (1.4-kb HindIII fragment of *A-51*, nucleotides 1235 to 2627 [numbered from the start of translation], GenBank accession number M65163), pSB1.0H (1.0-kb HindIII fragment of *B-51*, nucleotides 2592 to 3503, L04795), pSC1.5H (1.5-kb HindIII fragment of *C-51*, nucleotides 4257 to 5753, M65164), pSD1.4H (1.4-kb HindIII fragment of *D-51*, nucleotides 4566 to 6024, X85135), pT2C (alpha-tubulin gene, a gift from J. R. Preer, Jr., Indiana University [13], accession number X99490), and pBluescript (empty vector).

Northern and slot blot analyses. For Northern blots, RNA samples (5 to 10 µg) were separated in 1% SeaKem HGT agarose (BioWhittaker Molecular Applications) containing 6.7% (vol/vol) formaldehyde and 1× morpholinepropanesulfonic acid (MOPS) (pH 7.0). The gel was stained with a fresh staining solution (1 µg/ml ethidium bromide, 0.05 N NaOH) for 25 min and destained twice by 0.2 M sodium acetate (pH 4.0) for 20 min each time, and then pictures were taken under UV light. The gel was blotted onto a Hybond N+ filter (Amersham Biosciences) overnight, and the filter was UV cross-linked and then dried. For slot blots, 0.5-µg samples of plasmid solutions were dissolved in 0.4 M NaOH-10 mM EDTA (pH 8.2) and passed through a Hybond N+ filter. The filter was rinsed subsequently with 0.4 M NaOH and 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then air dried. These filters were incubated in a hybridization solution (1× Denhardt's solution, 0.02 M phosphate buffer, 5× SET [1× SET consists of 0.15 M NaCl, 0.03 M Tris, and 2 mM EDTA], 0.25% sodium dodecyl sulfate [SDS]) for 1 h at 65°C, then labeled probes were added, and the filters were incubated with shaking at 65°C overnight. The filter was washed three times for 30 min each time in a solution containing 0.2× SET, 0.1% SDS, 0.1% sodium pyrophosphate, and 25 mM phosphate buffer at 65°C or increasingly stringent temperatures (65°C, 68°C, and 70°C, respectively). Alternatively, prehybridization and hybridization were carried out in another hybridization solution (0.25 M phosphate buffer, 1 mM EDTA, 7% SDS, pH 7.2), then the filter was washed once for 15 min at 65°C in a solution containing 20 mM phosphate buffer (pH 7.2) and 1% SDS followed by three washes at 65°C or at increasing temperature as described above. Probes used are plasmid pSA8.8R containing the 8.1-kb EcoRI fragment of the *A-51* gene or a cloned 320-bp DNA fragment of IES6649 generated by PCR using a forward primer (5'-GGATAACATTAAATTCGGAG-3') and reverse primer (5'-ATATCAACAGGGCATTTC-3') with total genomic DNA from exconjugant cells. This reaction amplifies the circularized IES6649 of the *A-51* allele that is present transiently in genomic DNA of cells during sexual reproduction.

RESULTS

Isolation of A⁻ cell lines containing single-nucleotide mutations that result in termination or frameshift of the predicted A51 protein product. It is unknown how the surface antigen regulates expression of antigen genes in the nucleus. It is possible, for example, that a part of the A51 protein, such as the N-terminal portion, controls transcription of its own and other antigen genes. We believed that A⁻ mutants would be useful to investigate this expression system. Previously we reported mutants having single-nucleotide mutations in internal eliminated sequences (IESs) in the *A-51* gene (25, 26). IESs are germ line-specific sequences that are normally eliminated during programmed DNA rearrangement in the developmental process forming the somatic macronucleus (15, 17, 46). We used three *A* gene IES mutants (AIM2, AIM3, and AIM5), each of which has an insertion of a defective IES in different positions (positions 6649, 6435, and 1835, respectively, num-

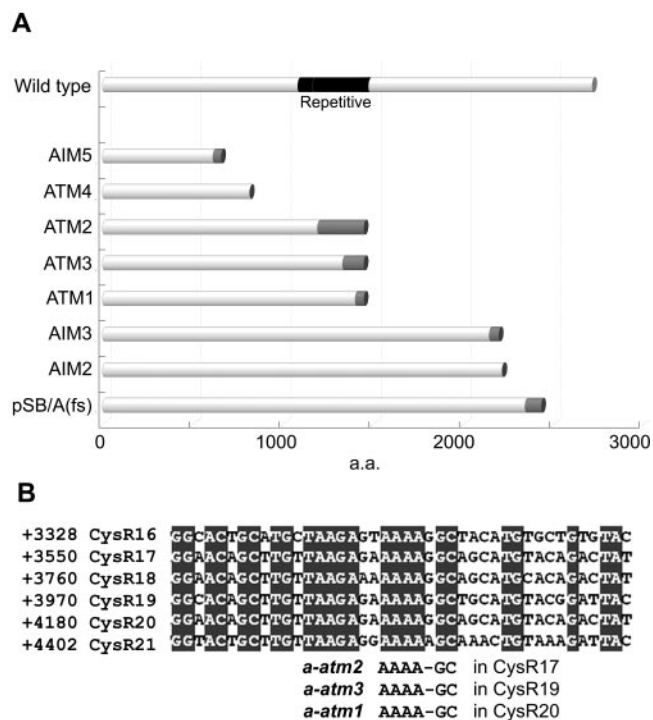


FIG. 1. Mutants used in this study. (A) Predicted protein products of the wild type and mutants are shown as a bar graph. Gray regions in the mutants represent the portion of the protein downstream of the frameshift or IES insertion sites whose amino acid sequences are altered relative to the wild-type protein. The black region in the wild-type protein indicates the repetitive region. The graph also shows the position of the frameshift in the pSB/A(fs) construct used in the previous study for comparison (41). a.a., amino acids. (B) Three deletion mutations of one of the two adjacent guanine residues in mutant alleles *a-atm1*, *a-atm2*, and *a-atm3* were located at exactly the same position in the repeats. Only 42 nucleotides in the 210- to 222-bp repeats are shown along with the positions numbered from the ATG start of translation. The direct repeats of the nucleotide sequence were aligned according to the cysteine repeat (CysR) found in the corresponding amino acid sequence (28).

bered from the start of translation) in the macronuclear *A-51* gene, resulting in termination or frameshift of the coding region (Fig. 1A) and thus the A⁻ phenotype (25, 26).

Additionally, we obtained new A⁻ cell lines derived from a screen for A51 expression of nitrosoguanidine-mutagenized cells. Test crosses showed that mutations in these A⁻ cell lines were recessive and mapped to the *A* locus (data not shown). Therefore, the 10.2-kb *A* locus in these A⁻ cell lines was directly sequenced from PCR products. One cell line contained a single-nucleotide substitution of G to A at position 2451 numbered from the start of translation, resulting in the introduction of a stop codon (TGA) at this position (this allele will be called *a-atm4* for *A*-gene truncation mutants). Three other mutants had single-nucleotide deletions of one of two successive guanine residues at positions 3573/4 (allele name *a-atm2*), 3993/4 (*a-atm3*), and 4203/4 (*a-atm1*), resulting in a frameshift of predicted protein products (Fig. 1A). Progeny from the crosses of these cell lines with the wild type were segregated into the A51 and A⁻ phenotype in the F₂ at the expected ratio for a single recessive allele (0.4 < *P* < 0.8). All

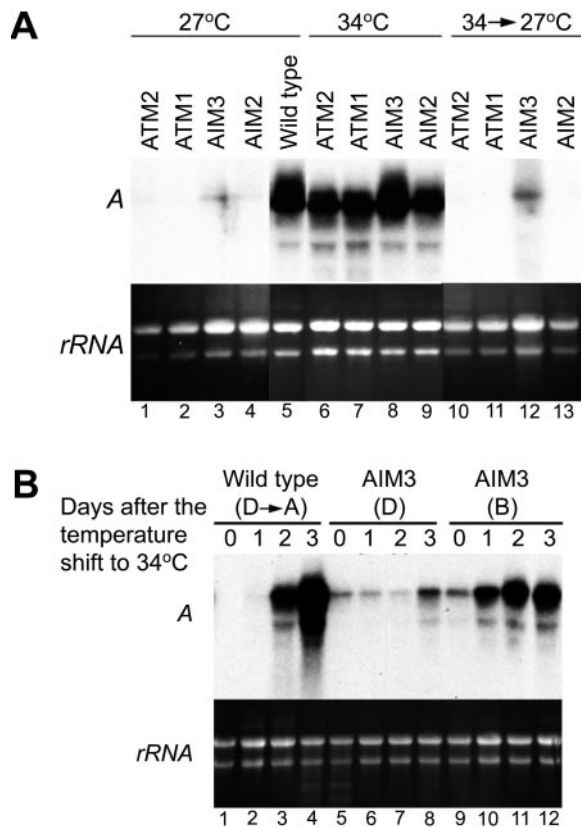


FIG. 2. Accumulation of mutant transcripts. (A) Northern blot of total RNA isolated from the mutant clones maintained at 27°C (lanes 1 to 4), 34°C for at least 2 days (lanes 6 to 9) and 27°C 2 days after culturing at 34°C (lanes 10 to 13). (B) Northern blot of total RNA isolated from the wild type and AIM3 strain after temperature shift from 27°C to 34°C for the indicated number of days. At day 0, cell cultures consisted of one antigen type and continued to be so in AIM3 mutants, but in wild-type culture, cells expressing A51 gradually increased after day 2. Ethidium bromide staining of rRNA was used as the loading control.

eight whole-cell PCR products from mutant F₂ progeny contained each respective mutation in the *A* locus, confirming that each single mutation is responsible for the A⁻ phenotype. Curiously, the frameshift mutations of *a-atm1*, *a-atm2*, and *a-atm3* were located at the same position in the central repeats of the *A-51* gene (Fig. 1B), suggesting that this region of the repeat unit is a mutation “hot spot.”

As expected, at 27°C, steady-state mRNA levels of the *A* gene were very low in the frameshift mutants analyzed in this report (Fig. 2A, lanes 1 to 5). Additional Northern analysis confirmed the absence of transcripts at 27°C in cell lines AIM5, ATM3 (*a-atm3/a-atm3*), and ATM4 (*a-atm4/a-atm4*) as well (data not shown).

In all these A⁻ mutants, other antigen expression appears normal. The D51 type was most commonly expressed, followed by the C51, B51, E51, and I51 types at 27°C, and the D51 and C51 types were predominant at 34°C (data not shown). Thus, the positions of the mutations in the *A* locus do not seem to affect the expression of other antigens. For the subsequent analyses, we present results using cell lines AIM2, AIM3, ATM1 (*a-atm1/a-atm1*), and ATM2 (*a-atm2/a-atm2*) which

predict protein products shown in Fig. 1A along with the frameshift construct pSB/A(fs) used in the previous study for comparison (41).

Uncoupling of the mutant transcript from the functional antigen at high temperatures. Expression of the A51 antigen is induced and maintained at 34°C overcoming expression of different antigen types (9). Steady-state *A-51* mRNA levels in wild-type cells are similar between 27°C and 34°C when the cells are expressing the A51 type (data not shown).

The mutants were tested for accumulation of the transcript at 34°C. The *A* transcript in all mutants was increased to a level similar to that in the wild type when they were grown at 34°C for at least 2 days, even though they are presenting other antigens on the cell surface (Fig. 2A, lanes 6 to 9). In a wild-type cell, expression of A51 normally excludes expression of other surface antigen genes. The *A-51* gene is the dominant antigen type at 34°C. Therefore, coexpression has not been observed previously at these conditions. The results demonstrate that the *A-51* gene is continually expressed at 34°C in the mutant and therefore a clear exception to mutual exclusion. We expect that this temperature induction is also true for wild-type cells, but coexpression of other antigens is prevented by the *A-51* gene product.

Since the cell lines used in Fig. 1A were mixtures of cells with different serotypes, pure cultures (one serotype) were examined for accumulation of the mutant *A* transcript. The results showed that the major contribution to the accumulation of *A* mRNA in the mutants was the temperature shift rather than changes of the expressed antigen types. AIM3 clones stably expressing either the D51 or B51 type were transferred to 34°C but still showed gradual accumulation of the transcript without switching the antigen type (Fig. 2B, lanes 5 to 12). Furthermore, the high *A* mRNA level in the mutants at 34°C was not maintained after the temperature shift back to 27°C (Fig. 2A, lanes 10 to 13). Apparently, high temperatures induced mutant *A* gene expression in the absence of the functional protein.

Expressed antigen types also had effects on *A* gene expression. The transcript levels for *A* mRNA in AIM3 cells were greater when the cell line expressed B51 antigen than when the cell line expressed D51 antigen (Fig. 2B, compare lanes 5 to 8 to lanes 9 to 12). Similar data were obtained from cultures grown at 27°C. Although there was a high variance in mRNA levels in different clonal lines, mutant *A* transcripts were commonly found in B51-expressing clones, whereas the transcripts were generally lower in abundance in D51-expressing clones (data not shown).

To determine whether the low levels of mutant transcripts at 27°C are controlled at the transcriptional level, wild-type and AIM2 cells were analyzed by a nuclear run-on transcriptional assay (Fig. 3). In wild-type cells, antigen gene transcription was tightly associated with the expressed antigen types on the cell surface (Fig. 3). In contrast, *A* run-on transcripts could not be detected in uninduced AIM2 cells at 27°C (Fig. 3). However, at 34°C, transcription of *A-51* and *D-51* occurred at similar levels (Fig. 3). These data showed that mutant mRNA levels were regulated at the transcriptional level.

The observations indicated that the transcriptional state of the mutant *A* alleles was uncoupled from the functional A51 protein at high temperatures. The results also suggested that

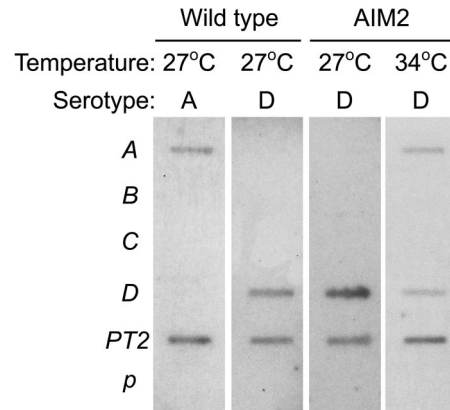


FIG. 3. Slot blots probed with labeled RNAs produced by in vitro run-on transcription. Each slot contains plasmid DNA from the 5' coding region of *A-51*, *B-51*, 3' coding region of *C-51* and *D-51* gene, as well as the alpha-tubulin gene (*PT2*) and an empty plasmid (*p*) as loading controls.

the frameshift mutants display defective maintenance of the transcriptional state at 27°C (Fig. 2A, lanes 10 to 13).

Expression of the functional *A* gene allowed stable transcription from the mutant allele. It is likely that transcriptional inactivity of the mutant *A* gene at 27°C was due to the absence of the functional *A* gene product in frameshift mutants. Therefore, we examined the effect of the functional *A* gene product on the mutant *A* gene using the *a-aim2* allele, which has a 341-bp IES insertion in the *A* gene (26).

The AIM2 strain expressing the D51 antigen was crossed with the wild-type strain. The resulting heterozygotes expressing the D51 type were grown at 27°C. At the same time, subcultures of the heterozygotes were grown at 34°C for 3 days to induce A51 expression. Cell lines containing 50 to 100% of cells expressing the A serotype were transferred back to 27°C and grown for 2 days. A Southern blot of total genomic DNA from these heterozygotes confirmed that both alleles were present in the somatic macronucleus (data not shown). Total RNA was extracted from D51- and A51-expressing heterozygotes and subjected to Northern blotting. Probing the blot with the IES that is present only in the mutant allele demonstrated that the mutant allele was transcribed in the heterozygotes only when the wild-type *A-51* allele was expressed (Fig. 4). Although the mRNA levels in the heterozygotes were less than those of AIM2 homozygotes grown at 34°C (4 to 15% of the total *A* transcripts), there are half as many *a-aim2* alleles in the heterozygotes and shifting to A expression may have been incomplete at 34°C. The data presented in Fig. 2 and 3 along with the results of this experiment show that the mutant allele was transcribed only in the presence of the wild-type *A-51* gene product at 27°C. This shows that the wild-type gene product is able to maintain transcription of the normally silenced mutant allele, consistent with the positive-feedback system for surface antigen expression in *Paramecium*.

DISCUSSION

The present paper described an analysis of a positive-feedback system for regulation of surface antigen gene expression

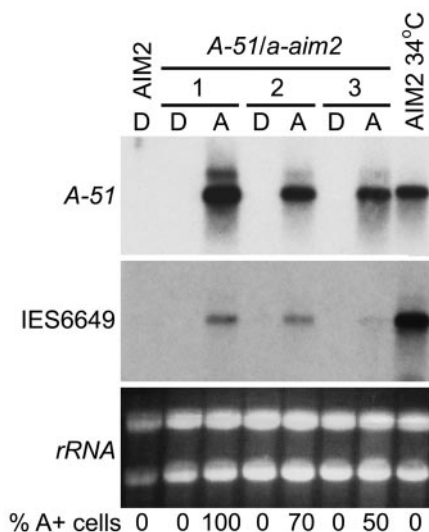


FIG. 4. Allelic expression in the heterozygous clones. Heterozygous clones (*A-51/a-aim2*) expressing the D51 type were grown at 27°C (lanes labeled “D”). Their subclones were simultaneously grown for 2 days at 34°C to induce A51 expression, then transferred back to 27°C, and grown for an additional 2 days (lanes labeled “A”). The percentage of A⁺ cells in each subclone at the time of RNA extraction is shown below each lane. A membrane containing total RNA from those three independent heterozygous clones was reprobbed with a plasmid containing the *A-51* gene after probing with a cloned DNA from IES6649 that is present only in the *a-aim2* allele. RNA from AIM2 grown at either 27°C (the leftmost lane) or 34°C (the rightmost lane) was presented as control. Ethidium bromide staining of ribosomal RNA (rRNA) was used as the sample loading control.

using termination and frameshift mutations of the *A-51* coding region. The results are consistent with a positive-feedback system allowing expression of the mutant alleles in the presence of the functional gene product.

Identification of a mutation “hot spot” in the *A-51* gene. We reported three new mutations in the *A-51* coding region which were found at exactly the same position in the repeat sequence (Fig. 1B). Considering the numerous possible positions that could result in a frameshift in the 8.2-kb *A-51* coding region, this region of the repeat unit may be a “hot spot” for deletion of a single nucleotide in the germ nucleus (micronucleus). Although we do not have a molecular explanation for these mutational hot spots, the fact that all three mutations were single-nucleotide deletions instead of substitutions suggests that slippage occurred upon DNA replication.

Temperature-dependent control of the transcriptional state of the *A* gene. The previous study using injection of a mutated *A* gene has shown that a single frameshift mutation in the coding region (Fig. 1A) reduced the rate of transcriptional activity of the corresponding gene at 27°C (41). In the present study, we further extended this observation with germ line point mutations at various positions in the *A-51* gene. The mutants did not show transcription of the *A* gene at 27°C (Fig. 2 and 3). However, since transcription of the *A* gene is repressed in wild-type cells expressing other antigens, the defect of the *A* gene mutants in its own expression is not clear by this observation alone.

We observed temperature-dependent *A* gene expression.

Mutants showed normal levels of *A* mRNA at 34°C (Fig. 2 and 3). Although temperature-dependent switching of surface antigens is well-known in ciliates, this is the first report that showed obligate derepression at the inducible temperature, which separated transcription from the functional surface antigen in *Paramecium*. The temperature-dependent derepression was gene specific (Fig. 3, rows B and C). Furthermore, no functional gene product was required for this activation, since the mutant alleles contained frameshifts in the coding region. The data are consistent if the *A-51* gene, possibly its promoter, contains an element that induces derepression at high temperatures. Thus, in the wild type, functional protein products should be delivered to the cell surface, suppress expression of other antigens, and ultimately replace the antigen type of the cell (Fig. 2B, lanes 1 to 4). On the other hand, the mutants cannot suppress the expression of other antigens, and this simply results in transcription without the functional surface antigen. This model is testable by measuring transcription of a construct containing part of the *A-51* gene, including the promoter region fused to the green fluorescent protein gene.

Expression of the wild-type gene maintains active transcription; evidence for a positive-feedback mechanism. Although the temperature-dependent control of antigen expression is simple, it does not explain clonally stable maintenance of the transcriptional state at 27°C, where transcription of the mutant alleles was not observed (Fig. 2 and 3). We showed that, at this temperature, a different mechanism was involved. The mutant allele in the heterozygotes with the wild-type allele was transcribed when the functional gene was expressed (Fig. 4). Therefore, the transcriptional state is likely controlled by the functional gene product. The results showed that positive feedback, a hypothesis that has been assumed for decades (3, 10, 19, 35), stabilizes expression of the normally silenced mutant allele. Thus, this is strong evidence supporting a positive-feedback mechanism for surface antigen expression.

Our observations on temperature-dependent derepression and maintenance of the derepressed state by positive feedback agree well with classical discussions on antigen switching. Sommerville (35) pointed out several decades ago; “it is generally considered that there are at least two stages in the control of immobilization-antigen synthesis; the initiation of expression of a locus in favorable physiological conditions by derepression and the continued stability of expression of that locus by self-induction.” If there are two such separate stages for antigen expression, our observations of *A* gene transcription in the mutants may correspond to the switching stage of antigen types, and the expression of the antigen types will be subsequently stabilized by their gene products.

Our interpretation also agrees with many earlier experiments. For example, Finger (10) added purified antigen protein to cells in an attempt to induce directed transformation of antigen types. The effect was not clear using cells with stable expression of antigen types. However, addition of antigen protein to cells with unstable antigen expression induced by puromycin enhanced the directed switching toward the added antigen types. This observation suggests a two-step switching of antigen types: (i) initial derepression and (ii) subsequent stabilization by self-regulation. Our observations of the frameshift mutants support this model. As shown by the temperature shift from 34°C to 27°C (Fig. 2A), the defect of the frameshift

mutants seems to be in maintenance of the active state of gene expression at 27°C. Thus, the wild-type gene product may have a role in stabilization rather than initial derepression of the corresponding gene.

Quantitative Northern analysis of the transcripts in the heterozygotes (Fig. 4) showed that the amount of the mutant transcript after shifting to 27°C from 34°C was less than that expected for full activation of the mutant allele in the heterozygotes (4 to 15% of the total *A* transcripts versus the expected 50%). However, the amount of the mutant transcript was still much higher than for the D51-expressing heterozygotes at 27°C where no transcript was observed (Fig. 4). One possible explanation for the lower than expected level of the mutant transcript is that the *a-aim2* allele was not recognized as the same gene as the *A-51* allele due to the 361-bp insertion in the 3' coding region. It is known that some combinations of *Paramecium* antigen alleles are allowed to express simultaneously in a single cell, while others show allelic exclusion of expression. The *a-aim2* allele may be close enough to the *A-51* allele to be stabilized by the wild-type gene expression, but its stabilizing effect may not be perfect, resulting in gradual reduction of the mutant transcript after the shift to 27°C. Another explanation for the reduced level of the mutant transcript may come from a recent finding that frameshift constructs of the *A-51* gene injected in the somatic nucleus at high copy numbers reduced the copy number of the corresponding gene in somatic nuclear development (12). The negative effect of a frameshift mutation at a high copy number seems to involve RNA-mediated gene silencing (12). We often observed a reduction in the copy number of the *a-aim2* allele in heterozygotes with *A-51* (data not shown), which may be consistent with the silencing effect of nonsense mutations. Thus, our experimental model might not be perfect, and there could be several distinct mechanisms that have affected gene expression from the mutant *a-aim2* allele.

The surface antigen may need to be presented on the cell surface to function in mutual exclusion. Nonsense-mediated mRNA decay (NMD) is a process which degrades mRNA molecules containing nonsense mutations (34, 43, 45). We have identified a few components of the NMD pathway in the preliminary *Paramecium* genome and observed that one of the NMD genes, *NMD3*, was required for vegetative growth at 27°C (unpublished results). The NMD pathway did not completely degrade mutant transcripts in *Paramecium*, since the presence of mutant *A* mRNA was correlated with transcriptional activity (Fig. 3).

Our results suggest that the molecule controlling positive feedback and mutual exclusion is not *A* mRNA. If this were true, then we would expect continued transcription of the *A* gene in the mutants at 27°C after induction of the mRNA at 34°C (Fig. 2A). Similarly, expression of other surface antigens (such as D51 in Fig. 3) should be shut down by accumulation of the mutant mRNA at 34°C by mutual exclusion, but instead we observed coexpression of surface antigen genes at this temperature. It is likely that the functional protein product is required for positive feedback and mutual exclusion of *A* antigen expression. It is highly unlikely that all mutant mRNAs are defective in the controlling functions, since most mutants analyzed in this report have single-nucleotide substitutions and the position of each substitution is located in various regions of

the *A* gene (Fig. 1). Although we do not deny all possible roles of RNA molecules transcribed from the *A* gene, our results favor the role of the protein product in the positive feedback and mutual exclusion in *A* gene expression.

Since mutant *A* transcripts were allowed to accumulate in some conditions (Fig. 2, 3, and 4), truncated *A* protein might be produced in the mutants. If this were true, regulatory activity of A51 antigen might be totally abolished by truncation of the C terminus. The C terminus is the position where protein is attached to the plasma membrane by the glycosylphosphatidylinositol anchor (4). Therefore, the surface antigen may be able to function only when presented on the cell surface.

We have observed some effect of the expressed antigen types on *A* gene mRNA levels in the mutants (Fig. 2B). Although there was a high variance in mRNA levels in different clonal lines, mutant *A* transcripts were more commonly found in B51-expressing clones and were generally lower in abundance in D51-expressing clones. Such differences in the level of *A* gene mRNA may be related to the higher identity of *A-51* with *B-51* (~65%) than *A-51* with *D-51* (~32%), although the mechanism for this effect is not clear.

It is unclear how the wild-type antigen controls transcription of surface antigen genes in the nucleus. Whereas the repertoire of antigen genes and conditions for their expression are different among wild stocks of *Paramecium*, earlier genetic analyses were unable to find any other genetic elements except the antigen alleles or loci themselves (38). Although mutational analysis has identified a few genes that are important for stable A51 antigen expression (9), no clear pathway has been identified so far for mutual exclusion in *Paramecium*. The results presented here suggest that a full-length *A* protein must be produced to stabilize transcription. It is possible that the protein must be presented on the cell surface to control mutual exclusion.

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