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Structure and Expression of Two Temperature-Specific Surface Proteins in the Ciliated Protozoan *Tetrahymena thermophila*

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The presence of specific proteins (known as immobilization antigens) on the surface of the ciliated protozoan *Tetrahymena thermophila* is under environmental regulation. There are five different classes (serotypes) of surface proteins which appear on the cell surface when *T. thermophila* is cultured under different conditions of temperature or incubation medium; three of these are temperature dependent. The appearance of these proteins on the cell surface is mutually exclusive. We used polyclonal antibodies raised against 30°C (designated SerH3)- and 40°C (designated SerT)-specific surface antigens to study their structure and expression. We showed that these surface proteins contain at least one disulfide bridge. On sodium dodecyl sulfate-denaturing polyacrylamide gels, the nonreduced 30°C- and 40°C-specific surface proteins migrated with molecular sizes of 69 and 36 kilodaltons, respectively. The reduced forms of the proteins migrated with molecular sizes of 58 and 30 kilodaltons, respectively. The synthesis of the surface proteins responded rapidly and with a time course similar to that of the incubation temperature. The synthesis of each surface protein was greatly reduced within 1 h and undetectable by 2 h after a shift to the temperature at which the protein is not expressed. Surface protein synthesis resumed by the end of 1 h after a shift to the temperature at which the protein is expressed. The temperature-dependent induction of these surface proteins appears to be dependent on the synthesis of new mRNA, as indicated by a sensitivity to actinomycin D. Surface protein syntheses were mutually exclusive except at a transition temperature. At 35°C both surface proteins were synthesized by a cell population. These data support the potential of this system as a model for the study of the effects of environmental factors on the genetic regulation of cell surface proteins.

Changes in gene expression in response to temperature shifts have been extensively documented. The heat shock response represents the major model system in eucaryotes in which environmental effects on gene regulation can be assessed. The heat shock response is ubiquitous in all organisms studied to date. It is manifested by the synthesis of 12 to 15 proteins whose expression can be brought about by a variety of environmental conditions (35). During the initial stages of a heat shock response, the synthesis of normal cell proteins is repressed. Depending on the organism, reexpression of normal cell proteins either can occur while the organism is still at the heat shock temperature or must wait until lower temperatures have been restored (17, 20, 23, 26, 27, 34). Although the function of these proteins is unknown, their importance to cell survival during a heat shock has been demonstrated (for a review, see reference 35). To better understand how environmental effects can cause changes in gene regulation, it would be advantageous to have a gene or gene family which reacts to a specific environmental stimulus without drastic effects on nonenvironmentally regulated genes.

The presence of specific proteins (known as immobilization antigens) on the surface of the ciliated protozoan *Tetrahymena thermophila* is under environmental regulation. Surface proteins are known as immobilization antigens because antisera raised against these proteins have the ability to stop cell movement when they are mixed with living *T. thermophila*. There are five different classes (serotypes) of proteins which appear on the cell surface when *T. thermophila* is cultured under differing conditions of temperature or incubation medium; three of these are temperature dependent. When cells are cultured at temperatures between 20 and 35°C the protein designated SerH is on the cell surface (28). If this same population of cells is shifted to temperatures below 20°C the protein designated SerL is expressed, whereas if the cells are raised to 36 to 40°C the protein designated SerT appears on the surface (22, 30). The appearance of these proteins are mutually exclusive; that is, when one protein is on the surface, all the others are undetectable (1, 31). In most of the work which supports this statement, antibodies were used to detect antigens on the cell surface by the immobilization of living, intact cells. Although considerable progress has been made in studying the SerH locus at a genetic level (7–10), little progress has been made in defining the product of this locus or in extending, to a molecular level, the observation that the syntheses of these proteins are mutually exclusive. Recently, Williams et al. (40) showed that a 30°C-specific surface protein (SerH3) was expressed as a result of shifting *T. thermophila* from 40 to 28°C. They showed that this protein consists of a single polypeptide with a molecular size of 52 kilodaltons (kDa). Doerder and Berkowitz (9a) showed that different alleles of the SerH locus (SerH1, SerH2, SerH3, and SerH4) produce proteins which vary in molecular size from 44 to 52 kDa.

These observations led us to investigate the structure and expression of two temperature-specific surface proteins expressed when *T. thermophila* is cultured at 30 or 40°C. We showed that both of these proteins contain at least one disulfide bridge, which contributes to some form of secondary structure. The presence of these surface antigens in total cell protein was dependent on the incubation temperature. A change in the incubation temperature resulted in a rapid modulation of surface protein synthesis. Experiments with actinomycin D indicated that RNA synthesis was required for the syntheses of both the 30°C- and 40°C-specific surface

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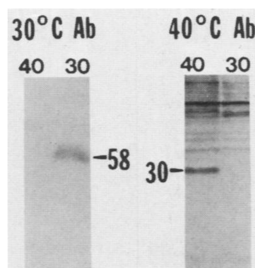

FIG. 1. Western blots of *T. thermophila* whole-cell protein reacted with 30°C-specific antibodies (30°C Ab) or 40°C-specific antibodies (40°C Ab). Whole-cell protein was electrophoresed on 7.5 or 12.5% acrylamide gels, blotted to nitrocellulose paper, and reacted with the appropriate antisera. 40, 40°C incubation temperature; 30, 30°C incubation temperature. The numbers along the side give the sizes of the antigenic bands in kilodaltons. Molecular sizes were determined by the migration relative to Bio-Rad Laboratories molecular weight standards.

proteins. The mutually exclusive expressions of the surface proteins were established beginning at 2 h after the shift in incubation temperature between 30 and 40°C. However, mutually exclusive expressions were not established in a population of cells incubated at a transition temperature. These results establish *T. thermophila* surface proteins and their genes as a potential model system to study how membrane protein composition changes in response to temperature.

MATERIALS AND METHODS

Cells and culture conditions. *T. thermophila* CU 355 was grown axenically in enriched proteose peptone (14) at 30 or 40°C. Strain CU 355 is homozygous for 30°C-specific surface protein expression (SerH3 [40]).

***T. thermophila* whole-cell protein preparation and cilia isolation.** Whole-cell protein was prepared as described by Guttman et al. (15). Briefly, 10^6 cells were pelleted, washed in 10 mM Tris (pH 7.4), and transferred to a 1.5-ml centrifuge tube. The pellet was lysed in 200 μl of sample buffer (0.05 M Tris [pH 6.8], 1.5% sodium dodecyl sulfate [SDS], 7.5% 2-mercaptoethanol, 0.75 mM phenylmethylsulfonyl fluoride) and placed in a boiling water bath for 3 min. The samples were stored at −20°C. This method was followed exactly for the preparation of nonreduced protein, except that 2-mercaptoethanol was deleted from the sample buffer. The cilia were isolated exactly as described by Calzone and Gorovsky (5).

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (24). All gels were composed of a 7.5 or 12.5% acrylamide resolving gel and 4% acrylamide stacking gel. The samples stored at −20°C were thawed on ice. Just before electrophoresis, 50 mg of crystalline urea was added to 65 μl of the sample and dissolved by vortexing at room temperature. Equal amounts of protein were electrophoresed in each lane of any particular gel for direct comparisons of the amounts of antigen present.

Antibodies and Western blotting. H serotype antibodies were provided by Peter Bruns. Their production has been previously described (4). These antibodies are specific for the 30°C-specific product of the SerH3 locus. Polyclonal antiserum containing 40°C-specific antibodies was generated by injecting rabbits intravenously with ca. 10^5 cells. *T. thermophila* was grown overnight at 40°C, and just before injection, the cells were washed into sterile phosphate-buffered saline. The rabbits were injected every other day for 2 weeks, followed by a lapse of 4 weeks. At the end of this period, serum samples were tested for 40°C-specific antibodies on Western blots of *T. thermophila* total cell protein. Rabbits were boosted by two more injections (administered every other day) of 40°C-specific cells.

Electrophoretic transfer and immunoblotting on nitrocellulose paper were performed by the procedures of Towbin et al. (37). The blots were incubated with antibody diluted in Blotto (21) for 12 h at room temperature. Detection of the primary antibody was by goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.) and was performed by the specifications of the manufacturer.

In some cases, surface protein antibodies were affinity purified from filter blots by modifications of the procedures of Olmsted (29). Total cell protein was blotted to nitrocellulose paper. After incubation with unfractionated anti-surface protein serum, the blots were washed extensively before the antibodies were released by treatment with 5 M sodium iodide in 10 mM Tris (pH 7.4)–150 mM sodium chloride. After dialysis against Tris-buffered saline, antibodies were concentrated and used on Western blots or for immunofluorescence of whole cells.

Immunofluorescence microscopy. All cells were fixed for indirect immunofluorescence as described by Wenkert and Allis (39). Briefly, 10 μl of fixative (saturated mercuric chloride–ethanol, 2:1) was added to 3 ml of cells (ca. 200,000 cells per ml) that had just been washed into 10 mM Tris (pH 7.4), and the cells were incubated at room temperature for ca. 5 min. The cells were washed twice in methanol, dropped directly onto cover slips, and allowed to air dry for ca. 30 min. The cells were incubated with preimmune or immune serum overnight at 4°C, washed extensively in Tris-buffered saline and incubated for ca. 30 min in fluorescein-conjugated swine anti-rabbit immunoglobulin G (Dako Corp., Santa Barbara, Calif.) at 37°C.

Immunoprecipitation of antigen. Cell protein was labeled with 15 μCi of [^{35}S]methionine per ml for 1 h. The cells were lysed in a solution containing 0.1 M Tris (pH 8.0), 0.2% SDS, and 4 mM EDTA and placed in a boiling water bath for 3 min, and an equal volume of a solution containing 0.1 M Tris (pH 8.0), 1% Nonidet P-40, and 1% deoxycholate was added. The samples were then incubated with anti-surface protein serum for 12 h at 4°C. The immune complexes were precipitated with protein A-Sepharose 4B beads according to the instructions of the manufacturer (Pharmacia, Inc., Piscataway, N.J.). The immune complexes were released from the beads by boiling in the SDS sample buffer and were electrophoresed on one-dimensional gels. The radioactive bands were visualized by impregnating the gel with En^{3}Hance (New England Nuclear Corp., Boston, Mass.) and exposing the dried gel to X-ray film at −80°C.

RESULTS

Characterization of anti-surface protein antibodies. To determine whether immune serum contained antibodies to temperature-specific proteins, total cell proteins from *T. thermophila* cultured at 30 or 40°C were subjected to one-dimensional polyacrylamide gels and analyzed by immunoblotting (Fig. 1). With the serum against the 30°C-specific protein, one major band was seen which was specific to cells cultured at 30°C. This band migrated with an apparent molecular size of ~58 kDa. For the 40°C serum, a large

number of bands were seen for cells cultured at 30 or 40°C. However, a prominent 30-kDa band was specific to cells cultured at 40°C. These results were expected since the serum against the 30°C-specific protein was raised against purified antigen, whereas the serum against the 40°C-specific protein was raised against intact cells.

The method by which the antisera were prepared should ensure that at least a portion of the antibodies recognize surface proteins. Cytological preparations of *T. thermophila* cultured at 40°C were stained with the appropriate antiserum by the indirect immunofluorescence method to determine whether the antisera recognized cell surface components. Unfractionated 40°C serum recognized cell surface components on cells cultured at 40°C, as indicated by the intensely immunostained cilia and cell periphery (Fig. 2B). To verify that the 30-kDa protein was a cell surface component, we isolated cilia from the cells cultured at 40°C. The proteins associated with this fraction were electrophoresed on one-dimensional gels and analyzed by immunoblotting. The 30-kDa protein was associated with cilia, indicating that this protein is a surface component (Fig. 2C). These data indicate that the antiserum against the 40°C-specific protein recognizes temperature-specific surface proteins in cells cultured at the appropriate temperature.

Surface protein structure. To determine whether the temperature-specific surface proteins consisted of single polypeptides or multiple disulfide-bridged subunits, total cellular proteins were prepared from cells cultured at 30 or 40°C under reduced or nonreduced conditions and were analyzed by immunoblotting. The nonreduced 30- and 40°C-specific surface proteins migrated as larger peptides than the peptides under reduced conditions (Fig. 3). The 30°C-specific surface protein from the nonreduced sample migrated with an apparent molecular size of ~69 kDa. When this sample was reduced, we detected a peptide of ~58 kDa. The nonreduced 40°C-specific surface protein had an apparent molecular size of ~36 kDa. When 2-mercaptoethanol was added to a portion of this sample, we detected a peptide of ~30 kDa. These results suggest two possibilities for the structure of the 30°C- and 40°C-specific surface proteins. Either the proteins exist as heterodimers consisting of at least two subunits joined by one or more disulfide bonds or an internal sulfhydryl bridge exists which produces a conformational constraint causing the protein to migrate differently in the presence of a reducing agent.

Time course of temperature-specific surface protein synthesis. *T. thermophila* cell cultures were shifted between 30 and 40°C to determine how rapidly synthesis of the surface proteins changed as a result of a change in the incubation temperature. Surface protein synthesis was monitored by radioactive labeling of total cell protein for 1 h with [^{35}S]methionine, immunoprecipitation with the appropriate antibody, and then one-dimensional SDS-polyacrylamide electrophoresis. Surface protein synthesis was extremely sensitive to incubation temperature (Fig. 4). The synthesis of the 30°C-specific surface protein was first detected beginning at 2 h after the cells were shifted from 40 to 30°C. In contrast, the synthesis of the 40°C-specific surface protein was undetectable beginning at 2 h after the cells were shifted in a similar manner. In cells shifted from 30 to 40°C, the situation was reversed, i.e., synthesis of the 40°C-specific surface protein was first detected beginning at 2 h after the shift in incubation temperature. The synthesis of the 30°C-specific surface protein was undetectable by the same time.

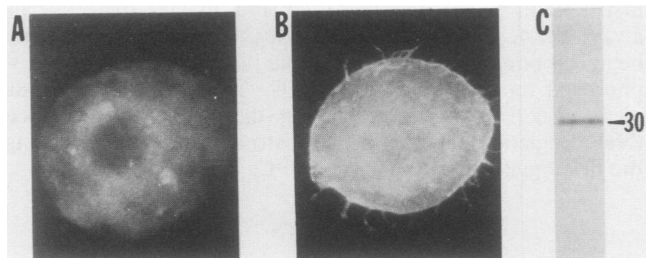

FIG. 2. Immunofluorescence microscopy (A and B) and electrophoresis (C) showing recognition by 40°C-specific antiserum of cell surface components. *T. thermophila* was grown overnight at 40°C and was prepared for immunofluorescence microscopy or was used for the isolation of ciliary proteins. The cells were incubated in preimmune serum (A) or 40°C-specific antiserum (B). The cilia were isolated from cells cultured at 40°C, electrophoresed on polyacrylamide gels, blotted to nitrocellulose paper, and incubated with 40°C-specific antiserum.

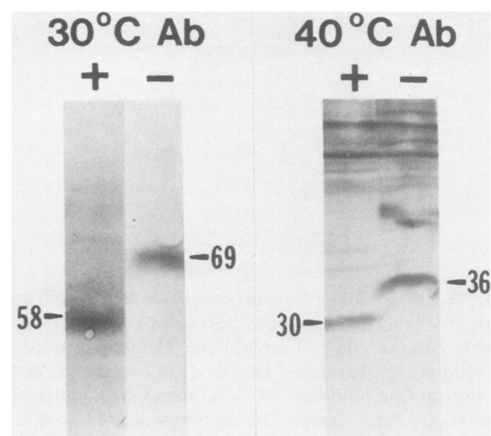

FIG. 3. Structure of *T. thermophila* surface proteins. Total cell protein from cells incubated at 30°C (30°C Ab) or 40°C (40°C Ab) was prepared in sample buffer containing (+) or deficient in (−) 2-mercaptoethanol. Total cell protein was electrophoresed, blotted to nitrocellulose paper, and incubated with the appropriate antiserum. Numbers along the side give the sizes of the antigenic bands in kilodaltons and were determined by their migrations relative to Bio-Rad molecular weight standards.

Effects of an RNA synthesis inhibitor on surface protein expression. A metabolic inhibitor was used to investigate the role of transcription in surface protein expression. It should be noted that specific metabolic inhibitors, such as the one used here, can affect cellular functions other than the one specified, and the results obtained must be interpreted with this in mind.

Actinomycin D was used to inhibit RNA synthesis as cells were shifted to different temperatures. The concentration of actinomycin D used (25 μg/ml) was shown to inhibit 96% of [^{3}H]uridine incorporation into acid-precipitable material in deciliated cells (16). Surface protein expression was followed by radioactively labeling cell protein with [^{35}S]methionine. The 30°C- or 40°C-specific surface protein was immunoprecipitated with the appropriate antibody, followed by one-dimensional SDS-polyacrylamide gel electrophoresis. The addition of actinomycin D to *T. thermophila* cultures from the onset of the temperature shift inhibited the synthesis of the 40°C- and 30°C-specific surface proteins (Fig. 5). Actinomycin D added to the cultures as late as 1 h after the onset of the temperature shift inhibited surface protein synthesis. If RNA synthesis was inhibited at later

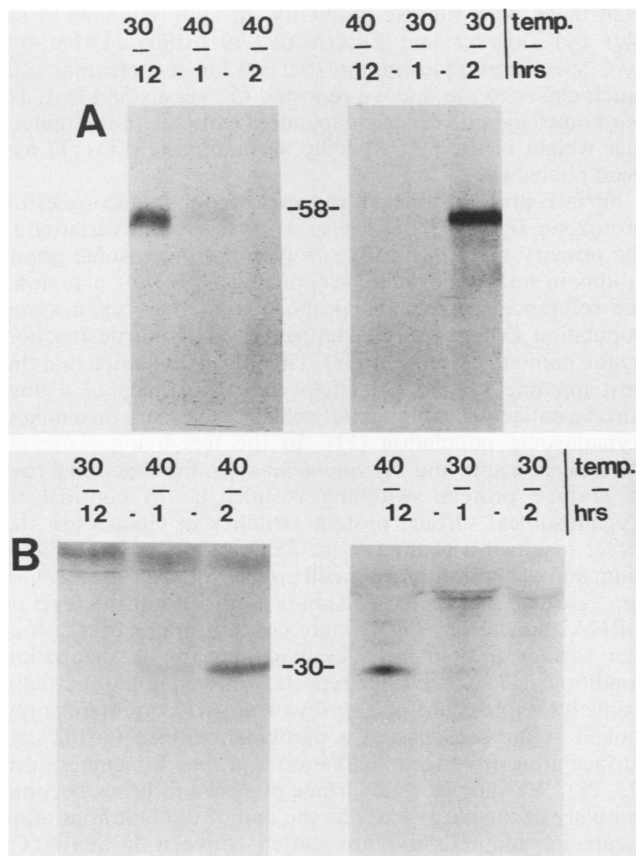

FIG. 4. Gel electrophoresis showing synthesis of temperature-specific surface proteins as cells are shifted between 30 and 40°C. Total cell protein was labeled with [^{35}S]methionine for 1 h before isolation. Surface proteins were immunoprecipitated with the appropriate antisera and electrophoresed on polyacrylamide gels. Numbers across the top represent incubation temperatures and hours at a particular temperature.

times after the temperature shift, no inhibition of surface protein synthesis was observed. These results are consistent with the notion that the transcription of a new mRNA within the first hour after a temperature shift is required for *T. thermophila* surface protein synthesis.

Mutually exclusive surface protein syntheses at transition temperatures. It has been reported by numerous investigators that appearance on the cell surface of *T. thermophila* immobilization antigens is mutually exclusive. The SerH antigen was on the surface of cells incubated at 20 to 35°C, while the SerT antigen was on the surface of cells incubated at 36 to 40°C (1, 22, 28, 30, 31). We wanted to determine whether one or both of the surface proteins was synthesized during transition temperatures. Cultures were grown at 33, 35, 37, or 39°C for 12 h. During the last hour, total cell protein was radioactively labeled by the addition of [^{35}S]methionine to the medium. Surface protein synthesis was monitored by immunoprecipitation with the appropriate antibody and one-dimensional SDS-polyacrylamide gel electrophoresis. The syntheses of the surface proteins were mutually exclusive at all temperatures except 35°C (Fig. 6). At 35°C, both the 30°C- and 40°C-specific surface proteins were synthesized. At all other temperatures tested, 30, 33, 37, 39, and 40°C (compare Fig. 4A and B for 30 and 40°C), only one of the proteins was synthesized in a cell population.

DISCUSSION

Surface protein structure. The structure of the temperature-specific *T. thermophila* surface proteins differs from that of other protozoan surface proteins studied. *Paramecium* surface proteins have molecular sizes ranging from 250 to 300 kDa. Each surface protein appears to consist of a single polypeptide chain (18, 19, 25, 33, 36). *Trypanosoma brucei* surface proteins have molecular sizes closer to those of *T. thermophila* (50 versus 58 kDa), but they lack disulfide bridges (6). However, Auffret and Turner (2) showed that trypanosome surface antigens exist in solution as noncovalently linked dimers and as higher oligomers. We showed that both the 30°C- and 40°C-specific surface proteins contain at least one disulfide bridge. This covalent modification can be involved in an intermolecular linkage with a smaller protein or an intramolecular linkage which places a conformational constraint on the protein. These possibilities remain to be resolved. However, we feel it is unlikely that migration differences observed for *T. thermophila* surface proteins, in the presence or absence of a reducing agent, are due to a 2-mercaptoethanol-activated protease, as observed for *Paramecium* surface proteins (36). We base this statement on the method of whole-cell protein preparation used (see Materials and Methods) and on the observations that conversion was complete and molecular sizes of the large subunit were constant from one protein preparation to the

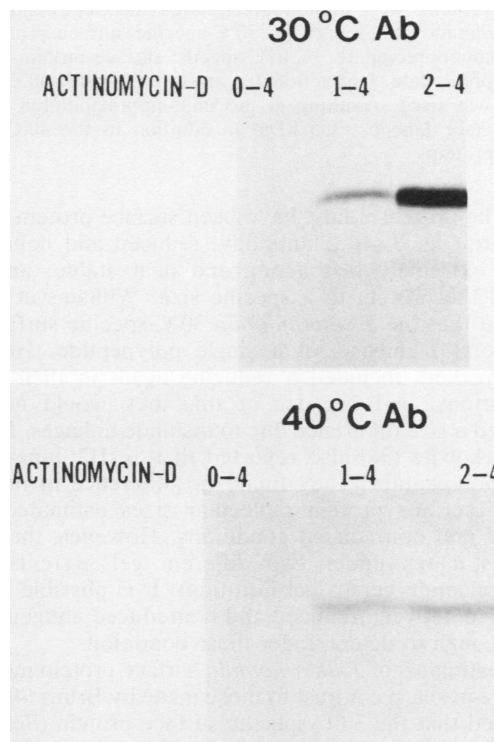

FIG. 5. Gel electrophoresis showing that surface protein synthesis is actinomycin D sensitive. Cells were incubated at 30 or 40°C for 12 h and shifted in the presence of actinomycin D to the alternate temperature. Cells were incubated with actinomycin D either from the onset (0 to 4) of the temperature shift or beginning 1 h (1 to 4) or 2 h (2 to 4) after the shift in incubation temperature. All samples were taken 4 h after the temperature shift. Total cell protein was labeled from the onset of the temperature shift with [^{35}S]methionine. Surface proteins were immunoprecipitated with the appropriate antisera and electrophoresed on acrylamide gels.

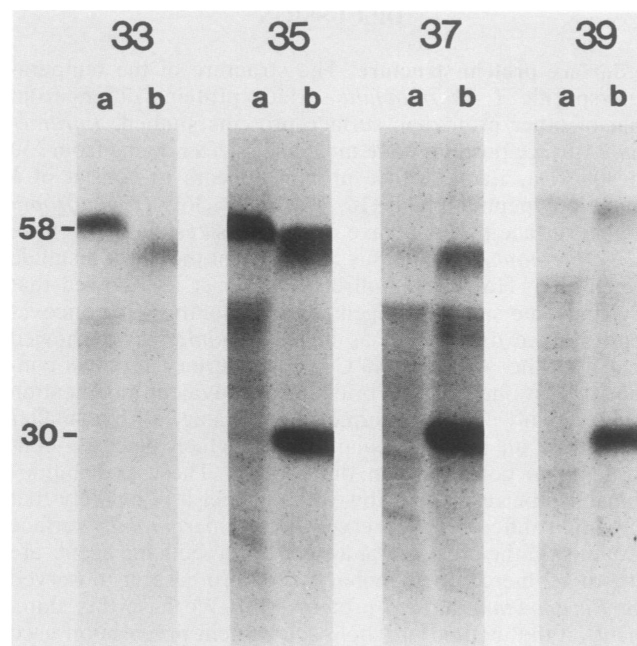

FIG. 6. Gel electrophoresis showing that surface protein syntheses are not mutually exclusive at a transition temperature. Cells were grown for 12 h at 33, 35, 37, or 39°C. During the last hour, whole-cell protein was radioactively labeled with [^{35}S]methionine. Surface proteins were immunoprecipitated and electrophoresed on polyacrylamide gels. Lanes: a, 30°C-specific surface protein antibody immunoprecipitate; b, 40°C-specific surface protein antibody immunoprecipitate. Unfractionated antiserum against 40°C-specific protein was used, resulting in the immunoprecipitation of other proteins (see lane b, ~55 kDa) in addition to the 40°C-specific surface protein.

next. The protease must have been surface protein specific (compare Fig. 3, 40°C antibody, reduced and nonreduced lanes), extremely fast acting and heat stable, and have cleaved the protein to a specific size. Williams et al. (40) reported that the *T. thermophila* 30°C-specific surface protein (SerH3) consists of a single polypeptide. However, these workers included a reducing agent in their sample preparations, and because of this they would not have observed a size difference due to disulfide linkages. Doerder and Berkowitz (9a) also reported that SerH3 consists of a single polypeptide. They found no difference in the 30°C-specific surface protein molecular sizes estimated under reduced and nonreduced conditions. However, they made their estimates under two different gel systems (SDS-polyacrylamide versus gel filtration). It is possible that the difference between reduced and nonreduced antigens is not large enough to detect under these conditions.

Our estimates of *T. thermophila* surface protein molecular sizes are in sharp contrast to those made by Bruns (4). Bruns estimated that the 30°C-specific surface protein (SerH) and the 40°C-specific surface protein (SerT) have molecular sizes of ~29 and ~23 kDa, respectively. The large differences between our estimates of the molecular sizes of these proteins and those of Bruns could be explained by the method of measurement. Bruns estimated molecular size by sedimentation through sucrose density gradients with a single molecular weight marker. In contrast, we estimated molecular size in denaturing SDS-polyacrylamide gels. Estimating molecular size by this method is not foolproof. However, this method probably gives more accurate results than those with sucrose gradients. In fact, Williams et al. (40) and Doerder and Berkowitz (9a) estimated that the 30°C-specific surface protein (SerH3) has a molecular size much closer to the one we reported (52 versus 58 kDa). To our knowledge, no other independent estimate of the molecular weight of the 40°C-specific surface protein (SerT) has been published.

Surface protein function and expression. In the parasitic protozoan *Trypanosoma brucei*, surface protein variation is the primary mechanism for survival of trypanosome populations in immunologically responding hosts (for a review, see reference 3). A small proportion of the trypanosome population switches surface antigens and avoids destruction by the immune system (11, 38). There is no evidence that the host immune system influences the appearance of a new surface antigen except to exert selective pressure on a mixed trypanosome population (12). In the free-living protozoa *Paramecium* spp. and *Tetrahymena* spp., the functional role of surface protein switching is unclear. In contrast to trypanosomes, surface protein switches in ciliates are the direct result of a change in incubation temperature or medium and occur in the entire cell population. In *Paramecium* spp., surface protein expression is controlled at the level of mRNA abundance. Steady-state concentrations of a particular surface protein mRNA are dependent on incubation conditions (13, 32). Earlier reports concerning the mutually exclusive expressions of *Tetrahymena* surface proteins only looked at the presence of a particular antigen on the cell surface after prolonged incubation at a specific temperature (22, 28). We showed that surface protein syntheses become mutually exclusive as early as the end of the first hour after incubation temperatures are shifted between 30 and 40°C. We implied that mutually exclusive expressions are due to the availability of newly transcribed surface protein RNA through the use of a metabolic inhibitor. At 35°C, mutually exclusive expressions appear to break down. Both the 30°C- and 40°C-specific surface proteins were synthesized in a single cell population at this transition temperature even after long (12 h) incubations. These results can be interpreted in two ways. Either the cell population consists of individuals producing both the 30°C- and 40°C-specific surface proteins or there are two subpopulations, one that produces the 40°C-specific surface protein and one that produces the 30°C-specific surface protein. Our results cannot distinguish between these possibilities. These results (a rapid, specific response by a population of cells to an easily manipulated environmental condition) indicate that the *T. thermophila* surface proteins and their corresponding genes are an excellent system in which to study membrane protein composition changes in response to temperature.

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