

Identification of λ gt11 Clones Encoding the Major Antigenic Determinants Expressed by *Theileria parva* Sporozoites†

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An antiserum, C16, was raised in cattle against freeze-thawed extracts of sporozoites of *Theileria parva* (Muguga). This antiserum, which neutralizes sporozoite infectivity *in vitro*, identified theileria-specific antigens having approximate molecular masses of 105, 90, 85, 69, 67, 52, 47, and 43 kilodaltons (kDa) on Western blots (immunoblots) of infected tick salivary gland extracts. The antiserum was used to screen an expression library of *T. parva* (Muguga) genomic DNA fragments. Three recombinant bacteriophage clones carrying different theilerial DNA inserts were studied. The expressed gene product from each clone was used to affinity purify antibodies from C16 antiserum for use in probing Western blots of uninfected and infected tick salivary gland extracts. The population of antibodies selected by each clone specifically recognized a subset of the antigens identified by C16 antiserum. The antigens fell into three distinct groups as defined by their reactivity with each set of selected antibodies. One group included antigens of 105, 90, 85, and 35 kDa, a second group included antigens of 69, 67, 52, 47, and 43 kDa, and the third group included an apparently distinct pair of antigens of 47 and 43 kDa. Thus, antibodies that reacted with determinants encoded by the three recombinant phage clones recognized all of the major antigens seen on Western blots probed with whole C16 antiserum. These results suggest that there may be only three immunodominant antigens expressed in *T. parva* (Muguga) sporozoites. Additionally, monoclonal antibodies have been raised which neutralize sporozoite infectivity *in vitro*. These antibodies react with epitopes of the antigens with M_r s of 69,000, 67,000, 52,000, 47,000, and 43,000 which are encoded in clone pgT-42 and have been used to localize these epitopes on the sporozoite surface.

The protozoan parasite *Theileria parva*, which is transmitted by the ixodid tick, *Rhipicephalus appendiculatus*, causes East Coast fever, a disease of cattle which continues to exert severe limitations on the development of the livestock industry in much of eastern and central Africa. Infection with *T. parva* is initiated when sporozoites are inoculated into the mammalian hosts by the tick during feeding. The sporozoites enter the lymphocyte, where they mature into schizonts. The intralymphocytic schizonts later develop to merozoites, which enter erythrocytes to become piroplasms, which infect ticks feeding on the parasitized host.

Although *T. parva* infection results in high mortality, some animals recover from the infection and are subsequently immune to homologous challenge. The occurrence of acquired immunity has encouraged the belief that a vaccine can be developed to protect domestic animals against East Coast fever. At present, it is possible to induce immunity by infecting cattle with sporozoites and simultaneously administering oxytetracycline (18, 19). However, this method of immunization affords protection against only a limited number of different *T. parva* stocks (2, 9).

It has been shown that animals which have developed immunity to *T. parva* exhibit antibody responses against proteins of the sporozoite stage (14) as well as a cell-mediated response against the schizont stage (5). There is evidence that antibodies raised against sporozoite antigens can block infectivity *in vitro* in a non-strain-specific manner (4, 15). However, the extent of protection conferred on cattle by this humoral response and the role played by

particular sporozoite antigens have not yet been reliably evaluated. Therefore, it is necessary to characterize more carefully both the humoral and cellular responses, to try to elicit comprehensive protective immunity against East Coast fever.

In this paper, we describe the characterization of three λ gt11 clones that encode all of the major antigenic determinants that are identified by antiserum directed against the sporozoite stage of the life cycle of *T. parva* subsp. *parva* (Muguga).

MATERIALS AND METHODS

Parasite stabilates. *T. parva* (Muguga) stabilate ILRAD 836 was used as the source of parasite material for all procedures described. For piroplasm preparation, calves (6 to 12 months of age) were infected by inoculation with a sporozoite stabilate prepared as previously described (2).

Bacterial strains and vectors. *Escherichia coli* Y1090 $r^- m^+$ and dephosphorylated λ gt11 arms were obtained from Promega Biotec (Madison, Wis.).

Preparation of tick salivary gland extracts. For the preparation of infected tick salivary gland extracts, nymphs from the ILRAD colony of *R. appendiculatus* were fed on cattle showing a piroplasm parasitemia after infection with *T. parva* (Muguga) stabilate 836. The nymphs were then allowed to molt, and the resultant adult *T. parva*-infected ticks were fed on rabbits for 4 days to induce the maturation of parasites to sporozoites within the salivary glands. The infected salivary glands were dissected out of the ticks and homogenized. The homogenate was centrifuged at $50 \times g$ for 5 min, and the supernatant, enriched for sporozoites, was passed over a DEAE column (Pharmacia Fine Chemicals, Uppsala, Sweden) by the method described elsewhere for isolation of sporozoites of *Plasmodium* spp. (11). The sporozoites were pelleted at $3,800 \times g$ for 15 min and stored as

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† This paper is dedicated to the memory of Jagruti Desai. Om shanti.

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a pellet at -70°C . For polyacrylamide gel electrophoresis, samples were prepared by suspension of 5 to 8 μg of protein in sample buffer containing 0.1 M Tris hydrochloride, pH 6.8, 2% sodium dodecyl sulfate, 2% β -mercaptoethanol, and 20% glycerol.

Extracts of uninfected salivary glands were produced either by freeze-thaw homogenization of whole, uninfected tick salivary glands or by procedures identical to those described above for infected glands, except that the nymphs were initially fed on uninfected cattle.

Preparation of lymphocyte homogenates. *T. parva* (Muguga)-infected lymphocyte line G6TPM was grown to a density of 10^6 cells ml^{-1} in L-15 medium which was supplemented with 10% fetal bovine serum, 1% tryptose phosphate broth, 0.5 μg of gentamicin ml^{-1} , and 2 μM glutamine. Cells were pelleted at $2,000 \times g$ for 10 min at 4°C , washed twice in saline, and suspended in homogenization buffer (0.25 M sucrose, 25 mM Tris hydrochloride, pH 7.4, 0.1 mM EDTA, 5 mM MgCl_2 , 5 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 8 μg of aprotinin ml^{-1}) at a density of 10^8 cells ml^{-1} . The suspension was freeze-thawed twice in liquid nitrogen and then was sonicated for 1 min. Insoluble components were then pelleted at $100,000 \times g$ for 30 min at 4°C . The pellet was suspended by vortexing in the original volume of homogenization buffer and sonicated. For polyacrylamide gel electrophoresis, equal quantities of the supernatant and pellet fractions were combined, an equal volume of $2\times$ sample buffer was added, and the mixture was boiled for 3 min before 60 μl of the mixture was loaded onto each gel lane.

Preparation of C16 antiserum. *T. parva* sporozoites isolated as described above were frozen and thawed four times in a minimal volume of distilled water and were sonicated for a total of 30 s on ice. Six calves, which had been screened for a lack of antibodies against theilerial antigens, were each immunized intramuscularly with 200 μg of the total protein of this extract in complete Freund adjuvant. Each animal was given three booster doses of 200 μg of total protein from the same preparation, at intervals of 2 weeks, in incomplete Freund adjuvant. Immune serum was collected 2 weeks after the last booster.

Immunoelectron microscopy of sporozoites. Monoclonal antibody TpM 12/18.15.8 was produced as described previously (7, 15, 20, 21). Thin cryosections of partially purified sporozoites were sectioned and labeled as described previously (16, 22).

Purification of *T. parva* (Muguga) piroplasms and piroplasm DNA. *T. parva* (Muguga) piroplasms were isolated as previously described (1). For purification of DNA, piroplasms were suspended in 10 ml of TNE (10 mM Tris hydrochloride, pH 7.8, 100 mM NaCl, 1 mM EDTA). Sodium dodecyl sulfate and RNase A (boiled in 10 mM Tris hydrochloride-0.1 mM EDTA, pH 8.0, for 10 min at 93°C) were added to final concentrations of 0.5% and 100 μg ml^{-1} , respectively. The suspension was incubated for 1 to 2 h at 37°C . Proteinase K (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was then added to a concentration of 100 μg ml^{-1} , and the preparation was incubated for an additional 2 to 3 h at 50°C . The lysate was extracted once with phenol, twice with phenol-chloroform (1:1), and twice with chloroform-isoamyl alcohol (24:1) before precipitation of DNA with 2 volumes of ice-cold 100% ethanol. DNA was pelleted at $2,500 \times g$ for 15 min at 4°C , dried at 37°C , and dissolved in sterile TE buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA).

DNA blotting. Fragments of DNA, digested with restriction enzymes, were resolved on 1% agarose gels and then

transferred onto nitrocellulose filters according to the method of Maniatis et al. (12). DNA was radiolabeled by nick translation with [α - ^{32}P]dATP ($>3,000$ Ci mmol^{-1} ; Amersham International plc, Amersham, United Kingdom) as described by Meinkoth and Wahl (13). Filter-bound DNA was hybridized with radiolabeled probes for 12 to 16 h at 65°C in a mixture containing $10\times$ Denhardt solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin), $4\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium PP_i (pH 7.4), and 0.1% sodium dodecyl sulfate. Filters were washed in two changes of $0.1\times$ SSC at 65°C for 30 min each and autoradiographed overnight on RX 100 X-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan) with a Cronex intensifying screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.).

Construction of genomic expression libraries in $\lambda\text{gt}11$. A library of *T. parva* (Muguga) genomic DNA fragments was constructed in the bacteriophage vector $\lambda\text{gt}11$ by methods previously described (23). Approximately 2 μg of piroplasm DNA was sheared by passage through a 19-gauge needle, 250 to 300 times, to produce fragments of 3 to 8 kilobases (kb) in size. The DNA was methylated with *EcoRI* methylase by using reaction conditions described by the manufacturer (New England Biolabs, Inc., Beverly, Mass.). Treatment with a Klenow fragment of DNA polymerase and ligation with *EcoRI* linkers were carried out as described elsewhere (12). The excess linkers underwent digestion twice, for 2 h each time at 37°C , with 100 U of *EcoRI*, followed each time by phenol-chloroform (1:1) extraction and passage through a Sephacryl S-300 spin column (Pharmacia). The DNA fragments were then mixed with 0.5 μg of dephosphorylated $\lambda\text{gt}11$ arms at a 1:2 molar ratio of inserts to arms and ligated with T4 DNA ligase. The recombinant DNA was then packaged into phage particles by using commercially available extracts (Promega Biotec), and the resultant phage was amplified by plating on *E. coli* Y1090. Approximately 1.1×10^6 recombinant phage were produced from 0.1 μg of insert DNA in a library that contained 85% recombinants.

Immunoscreening of $\lambda\text{gt}11$ expression libraries. Immunoscreening was carried out as described by Young et al. (23). Y1090 cells were grown to the logarithmic phase in L broth containing 100 μg of ampicillin ml^{-1} , pelleted by centrifugation at $2,500 \times g$ for 10 min at 4°C , and suspended in a 1/10 volume of 10 mM MgSO_4 . Phage particles (4×10^4) were inoculated into each 200 μl of cells and incubated at 37°C for 5 min. Cells and phage were then mixed with 8 ml of top agar, spread on 150-mm plates containing NZYCM agar, and incubated for 4 h at 42°C until plaques were clearly discernible. Nitrocellulose filters, previously soaked in 10 mM isopropyl- β -D-thiogalactopyranoside and dried, were then overlaid on the plates, and the incubation was continued at 37°C for a further 3 h. Filters were then washed briefly in TS buffer (10 mM Tris hydrochloride, pH 8.0, 150 mM NaCl), blocked with TS containing 5% ovalbumin for 15 min at 23°C , and finally incubated in a 1:160 dilution of antiserum in TS-5% ovalbumin overnight at 23°C . Filters were then washed for 5 min in each of two changes of TS, two changes of TS-0.001% Nonidet P-40, and two changes of TS. Each filter was then immersed in TS-5% ovalbumin containing rabbit anti-bovine antibody conjugated to horseradish peroxidase (ICN Immunobiologicals, Lisle, Ill.) and incubated at room temperature for 2 to 5 h. Six washes were again carried out as described above. The filters were then developed in a mixture containing 10 mM imidazole, pH 7.4, 0.25 mg of dianisidine *o*-dihydrochloride (Sigma Chemical Co., St. Louis, Mo.) ml^{-1} , and 0.37% hydrogen peroxide.

Plaques showing positive signals were picked and re-screened three times.

Affinity purification of antibodies. Antibodies were selected by using the modification of Ozaki (17) of the original method of Hall et al. (6). Neutralization of 5 ml of 0.2 M glycine following antibody elution was carried out by the addition of 850 μ l of a mixture containing 350 μ l of 2 M Tris, pH 10.2, 500 μ l of 10 \times TS, 5 μ l of 10% NaN₃, and 0.25 g of ovalbumin.

Sodium dodecyl sulfate-polyacrylamide gels and Western blotting. Samples were resolved on polyacrylamide gels as described by Laemmli (10). Seven and one-half percent acrylamide gels were employed for the resolution of proteins in salivary gland and lymphocyte extracts. Western blotting (immunoblotting) was performed with a Trans-Blot cell according to the specifications of the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). Western blots were probed with antisera in a manner identical to that used for the immunological screens described above, with the exception that visualization of antigen-antibody complexes was accomplished by incubation of each filter in 20 ml of TS containing 1 μ Ci of ¹²⁵I-labeled protein G (Amersham) for 1 h at room temperature followed by six washes and overnight exposure to RX 100 X-ray film (Fuji) at -70°C with a Cronex intensifying screen (du Pont).

RESULTS

Western blot analysis of theileria-specific antigens. Antisera were raised in six different calves by immunization with extracts of freeze-thawed *T. parva* (Muguga) sporozoites. These antisera neutralized sporozoite infectivity in vitro (A. J. Musoke, unpublished data) and were therefore considered suitable for use in identifying potentially protective antigens. All six of the antiserum specimens reacted with identical theileria-specific antigens, with variations occurring only in individual serum antibody titers.

Figure 1A shows a typical Western blot of uninfected (lane U) and infected (lane I) tick salivary gland extracts probed with one of the antiserum specimens, C16. The uninfected gland extracts were crude homogenates of tick salivary glands, whereas the infected glands were passed through DEAE-cellulose columns to enrich for sporozoites. Eight bands, having approximate molecular masses of 105, 90, 85, 69, 67, 52, 47, and 43 kilodaltons (kDa) and which were specific for the infected salivary gland extract, were identified. Theileria-specific antigens under 40 kDa in size could not be unequivocally identified on these blots because they were masked by strong signals seen in the uninfected extracts (Fig. 1A, lane U).

Antiserum C16 was also tested for cross-reactivity with proteins expressed in the intralymphocytic macroschizont stage of the life cycle. Patterns produced by probing uninfected (lane U) and macroschizont-infected (lane I) lymphocyte homogenates are shown in Fig. 1B. Some reactivity of C16 antiserum with schizont proteins was seen, with signals detectable in the molecular size range from 105 to 50 kDa.

Identification of clones encoding sporozoite antigens. Antiserum C16 was next employed to screen a λ gt11 expression library of *T. parva* (Muguga) genomic DNA fragments. By screening 3 \times 10⁵ phage plaques, 12 clones were identified and carried through two rounds of plaque purification. Upon analysis, the 12 clones were found to contain five unique DNA sequences. The expressed fusion product from each clone was used to affinity purify antibodies from C16 antiserum. The population of antibodies resulting from each af-

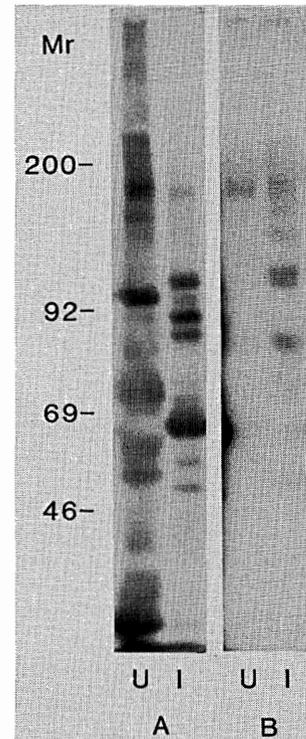


FIG. 1. Western blots probed with anti-sporozoite antiserum C16. (A) Uninfected (U) and sporozoite-infected (I) tick salivary gland extracts; (B) uninfected (U) and macroschizont-infected (I) lymphocyte homogenates. Antigen-antibody complexes were visualized by using ¹²⁵I-conjugated protein G. Sizes (10³) of the *M_r* markers are indicated on the left.

finity purification was next applied to a Western blot of infected and uninfected tick salivary gland extracts to identify proteins displaying determinants encoded by each clone. Three of the five clones, λ gTpS-17, λ gTpS-42, and λ gTpS-13, also referred to as 17, 42, and 13, respectively, selected antibodies that reacted with antigens expressed at the sporozoite stage of the *T. parva* life cycle. These clones were chosen for further characterization.

Figure 2A shows a Western blot of uninfected (lane U) and infected (lane I) salivary gland extracts probed with whole antiserum C16. In these experiments, infected and uninfected salivary preparations were prepared identically by passage through DEAE-cellulose. This served to enrich the infected preparations for sporozoites while reducing the representation of tick antigens in both homogenates. Western blots identical to that in Fig. 2A were probed with antibodies that were affinity purified by the fusion proteins of the three clones. Antibodies selected by clone 17 (Fig. 2B) reacted with four proteins having molecular masses of 105, 90, 85, and 35 kDa, indicating that clone 17 encodes determinants that are common to this group of proteins. These selected antibodies also revealed that the 35-kDa antigen is a theileria-specific molecule, an observation that was not clearly apparent from blots probed with the C16 antiserum. Clone 42 (Fig. 2C) selected antibodies that identified proteins having molecular masses of 69, 67, 52, 47, and 43 kDa, defining a second group of proteins carrying common determinants that are distinct from those identified by clone 17. Antibodies purified by clone 13 (Fig. 2D) identified a pair of proteins having molecular sizes of 47 and 43 kDa. This last

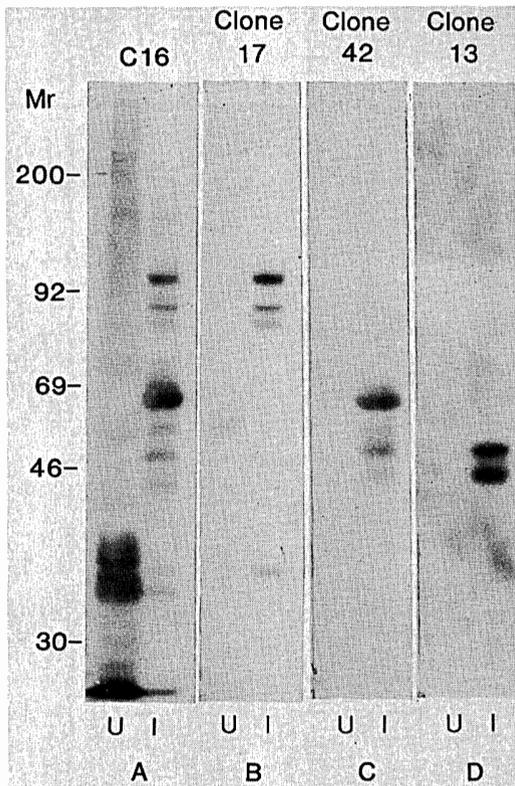


FIG. 2. Western-blotted protein patterns of uninfected (lanes U) and infected (lanes I) tick salivary gland extracts probed with C16 antiserum (A) and with affinity-purified antibodies against clone 17 (B), clone 42 (C), and clone 13 (D). Sizes (10^3) of the M_r markers are indicated on the left.

pair of antigens were of the same sizes as proteins identified by clone 42 but did not contain determinants in common with the 69-, 67-, or 52-kDa antigens and are, therefore, considered a distinct group. Thus, the determinants encoded by clones 17, 42, and 13 define three groups of antigens. The 11 proteins included in these three immunologically defined groups account for all of the major theileria-specific antigens identified by C16 antiserum on Western blots of theileria-infected tick salivary gland extracts. These results exclude sporozoite proteins less than 35 kDa in size, which would not be resolved by the gel system used.

Hybridization of cloned DNA fragments to Southern blots of *T. parva* DNA. Each of the three clones (17, 42, and 13) was employed to probe *EcoRI* and *BamHI* digests of purified *T. parva* (Muguga) piroplasm DNA. Figure 3, lane 1, shows an ethidium bromide-stained agarose gel pattern of *T. parva* DNA digested with *EcoRI*. Lanes 2, 3, and 4 of this figure show the hybridization patterns produced by probing identical patterns with radiolabeled clones 17, 42, and 13, respectively. None of the clones identified restriction fragments of the same size, indicating that each of the three DNA fragments contains nonoverlapping sequences. With clone 17, a 9.3-kb DNA fragment was observed; with clone 42, two fragments of 2.3 and 0.3 kb were observed; and with clone 13, a fragment of 3.3 kb was observed. Figure 3, lane 5, shows an ethidium bromide-stained agarose gel pattern of *BamHI*-digested *T. parva* (Muguga) piroplasm DNA. Hybridization of the three clones (17, 42, and 13) to different *BamHI* restriction fragments (lanes 6, 7, and 8, respectively) confirmed that the three clones contained DNA inserts that

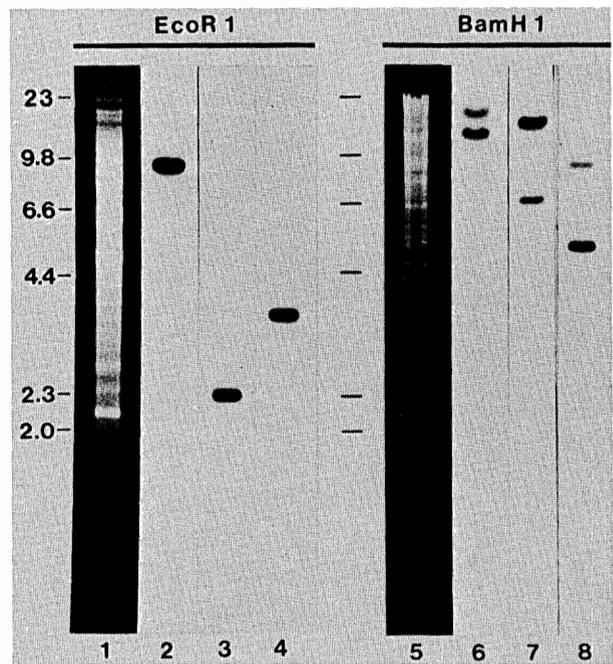


FIG. 3. Restriction patterns produced by digestion of *T. parva* (Muguga) piroplasm DNAs which were probed with radiolabeled clones λ GpS-17, λ GpS-42, and λ GpS-13. Ethidium bromide-stained agarose gels of *EcoRI*- and *BamHI*-digested DNAs are shown in lanes 1 and 5, respectively. Blotted patterns digested with *EcoRI* (lanes 2 to 4) and *BamHI* (lanes 6 to 8) were probed with radiolabeled clone 17 (lanes 2 and 6), clone 42 (lanes 3 and 7), and clone 13 (lanes 4 and 8). In addition to the strong signals evident in each lane, faint signals representing hybridization to fragments having approximate sizes of 0.3 and 0.6 kb also occurred in lanes 3 and 6, respectively. DNA size markers, in kilobases, are indicated to the left of the figure.

were localized on different restriction fragments. Two of the clones, 13 and 42, identified two *BamHI* fragments, indicating the presence of a single *BamHI* restriction site within each cloned fragment, while clone 17 identified three fragments, indicating the presence of two *BamHI* sites within the molecule.

Restriction maps of clones 17, 42, and 13. Inserts from each of the three clones (λ GpS-17, λ GpS-42, and λ GpS-13) were released by digestion with the restriction enzyme *EcoRI* and sized on an agarose gel. Clone 17 released a DNA fragment 5.0 kb in length, while clone 42 released two fragments, indicating the presence of an internal *EcoRI* site, having insert sizes of 2.3 and 0.3 kb. Clone 13 released a single 2.1-kb DNA insert. The presence of the internal *EcoRI* site within clone 42 is consistent with its hybridization to two restriction fragments on Southern blots of *EcoRI*-digested piroplasm DNA (Fig. 3).

Each of the four *EcoRI* restriction fragments was transferred into the plasmid vector pUC-18, and the four resulting clones were designated pgT-17, pgT-42L (containing the large, 2.3-kb *EcoRI* fragment), pgT-42S (containing the small, 0.3-kb *EcoRI* fragment), and pgT-13. Restriction maps were next constructed for the cloned DNAs. The clones were digested with enzymes *KpnI*, *BamHI*, *HindIII*, *EcoRI*, *AccI*, and *HincII*. Restriction patterns produced by agarose gel electrophoresis were then employed to construct the maps for each DNA insert. Shown in Fig. 4 are the restric-

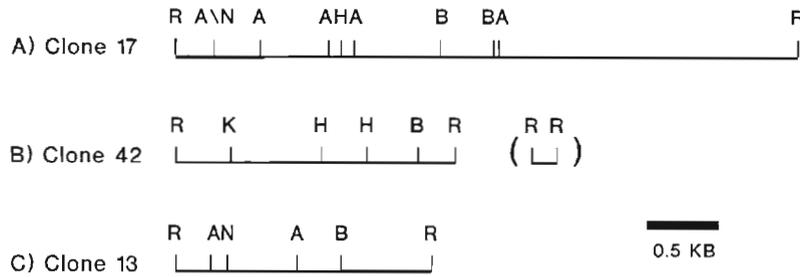


FIG. 4. Restriction maps of the theilerial DNA inserts in plasmid clones pgT-17 (A), pgT-42L and pgT-42S (B), and pgT-13 (C). Maps display the locations of restriction sites for the enzymes *EcoRI* (R), *BamHI* (B), *HindIII* (H), *AccI* (A), *HincII* (N), and *KpnI* (K). The 0.3-kb fragment contained in clone pgT-42S (shown in parentheses) was not restriction mapped.

tion maps for clones pgT-17 (A), pgT-42L (B), and pgT-13 (C). The 0.3-kb insert of pgT-42S is designated in parentheses (B) but because of its small size was not mapped. It can be seen that the number of internal *BamHI* sites identified in each DNA insert is also consistent with the number of restriction fragments of *BamHI*-digested piroplasm DNA that hybridized with each probe in Fig. 2. Clones 42 and 13 contained portions of two *BamHI* fragments, while clone 17 encoded portions of three fragments. Additionally, it is clear that each clone exhibited a unique restriction map, further confirming that the three clones encoded different regions of the *Theileria* genome.

Localization of epitopes encoded by clone pgT-42. Monoclonal antibodies have been raised against sporozoite antigens by immunization of mice with partially purified sporozoites (15). Antibodies resulting from the fusions reacted with those epitopes encoded by the group of antigenically related proteins having M_r s of 69,000, 67,000, 52,000, 47,000, and 43,000. These epitopes were encoded in clone pgT-42. One of these monoclonal antibodies, TpM 12/18.15.8, was employed to localize the epitopes common to this group of proteins on sections of *T. parva* sporozoites. Shown in Fig. 5 is an electron micrograph of a thin section of sporozoite with antigen-antibody complexes visualized by using protein A-gold. Complexes can be seen localized to the outer coat of the parasite. None of the battery of monoclonal antibodies produced by this immunization protocol reacted to the other two groups of antigens.

DISCUSSION

We have identified 11 theileria-specific proteins, ranging in molecular size from 35 to 105 kDa, that react with the serum of a calf inoculated with freeze-thawed extracts of sporozoites. This serum, C16, is one of a group of antisera that have been found to neutralize the infectivity of sporozoites in vitro and therefore reacts with antigens that may be of value in the development of an ant sporozoite vaccine.

Antiserum C16 was also found to react with a number of antigens expressed in the macroschizont. This reactivity could result from an expression of some of the antigens in both stages of the life cycle. For instance, a 105-kDa antigen is present in both salivary gland and infected lymphocyte preparations. However, we have not been able to react antibodies selected by clone λ GpS-17 with schizont antigens, suggesting that the 105-kDa antigens in sporozoites and schizonts are different proteins. It is also possible that antigens recognized in the schizont patterns are stage specific but contain determinants that are cross-reactive with sporozoite antigens. Such cross-reactivity has been observed previously in studies of the malaria life cycle (8).

Screening a λ gt11 expression library of *T. parva* genomic DNA with antiserum C16 yielded three recombinant phage clones encoding portions of theileria-specific antigens. Antibodies directed against the determinants encoded by these clones define three groups of antigens. The 105-, 90-, 85-, and 35-kDa proteins are included in one group. The 69-, 67-, 52-, 47-, and 43-kDa proteins are included in a second distinct group, while the third group is made up of the 47- and 43-kDa proteins. The reactivity of more than one protein with antibodies selected by a single cloned DNA fragment could indicate that the smaller proteins within each of the

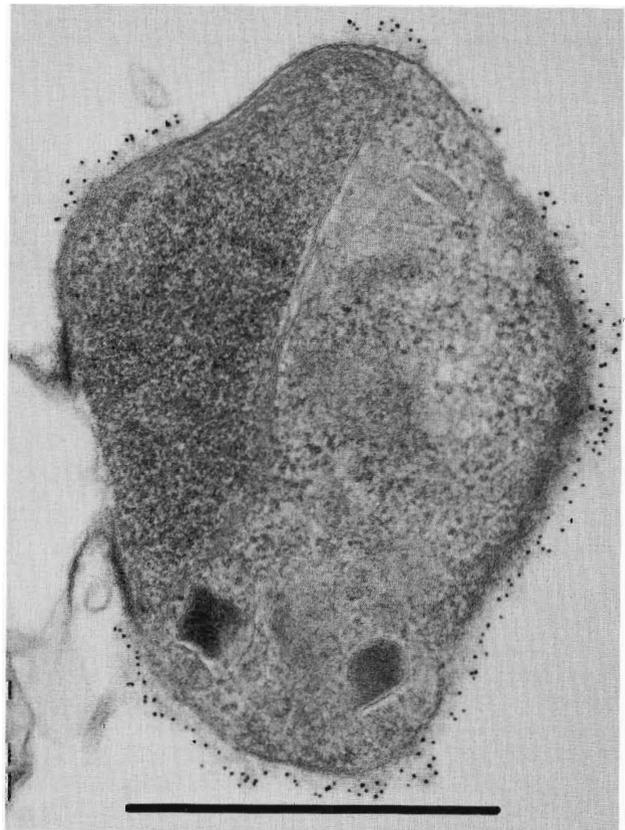


FIG. 5. Localization of epitopes encoded in clone pgT-42 on sections of *T. parva* sporozoites. Monoclonal antibody TpM 12/18.15.6, which reacts with the subset of proteins having M_r s of 69,000, 67,000, 52,000, 47,000, and 43,000, was used to localize the epitopes encoded within plasmid pgT-42 on thin sections of sporozoites. Antigen-antibody complexes were then visualized by using protein A-gold. Bar, 1 μ m.

three groups are derived from a primary antigen by either degradation or processing activities. There is evidence for this type of relationship among proteins identified by antibodies to clone 17 (K. P. Iams, Mol. Biochem. Parasitol., in press). If the antigens in each group proved to be related in this manner, it would mean that only three immunodominant proteins occur in the sporozoite. However, additional explanations for these results are possible. It could be that several proteins which are the products of different genes display common or cross-reacting antigenic determinants. This proposition is contradicted by the fact that in characterizing the 12 λ gt11 clones identified with antiserum C16, only those DNA inserts showing homology with clones 17, 42, and 13 selected antibodies to their respective antigenic groups. A final possibility is that more than one gene product could be encoded and expressed by each of the cloned DNA fragments. This alternative would require transcription of multiple mRNAs from the cloned DNA inserts with translation initiated from parasite control sequences—an unlikely occurrence, but one that cannot yet be excluded. Our favored interpretation of these results is that each of the three antigenic groups of proteins actually includes a single antigen and its breakdown or processed products.

Characterization of the DNA inserts in the three phage clones confirms that each is carrying a different portion of the *Theileria* genome, as evidenced by the unique hybridization patterns produced on *Eco*RI and *Bam*HI restriction patterns and the variation in their restriction maps. It is now necessary to localize and sequence the coding regions for each of the genes as well as attempt to localize the antigenic regions against which antibodies react. This work is now under way.

One puzzling aspect of these results was the unequal reactivity of C16 antiserum with tick antigens in uninfected and infected salivary gland extracts shown in Fig. 2. This occurred despite the fact that the two extracts were prepared by identical procedures. One explanation for this phenomenon is that theilerial proteins represent a significant amount of the material present in the infected tick salivary gland extracts and thus reduce the representation of tick proteins. It is also possible that *T. parva*-infected cells change their physiological properties in such a way as to cause alterations in the expression of certain tick proteins.

Previous work carried out with the group of antigenically related proteins having M_r s of 69,000, 67,000, 52,000, 47,000, and 43,000 suggests a role for the epitopes encoded in these antigens in eliciting antibodies that neutralize sporozoite infectivity in assays *in vitro* (4, 14, 15). Localization of this antigen on the parasite surface by using monoclonal antibodies has also been described previously (3) and correlates with the results we describe here. We have not yet been able to localize the other two groups of epitopes encoded in λ gTpS-13 and λ gTpS-17 in the sporozoite. This is due to our inability to obtain monoclonal antibodies reactive with these antigens. We have also found that neither of the two other clones produces a fusion product, making it difficult to obtain antigen for immunization. Other studies by one of us have shown that the expression of *T. parva* genes unfused to the *lacZ* gene is not an uncommon occurrence (data not shown). It will therefore be necessary to fragment and reclone the two remaining clones in order to obtain a purifiable fusion product for the production of antisera. This work is now under way.

By studying the three cloned theilerial DNA fragments contained within λ gTpS-17, λ gTpS-42, and λ gTpS-13, it has been possible to define the antigenic relationship among all

of the major sporozoite antigens identified by antiserum C16. Use of affinity-purified antibodies from each fusion protein suggests that as few as three sporozoite antigens express all of the determinants against which the bovine immune system responds. One of these cloned DNAs encodes epitopes which elicit antibodies that block sporozoite infectivity *in vitro*. With the acquisition of gene sequences encoding all of these determinants, it is now possible to rigorously test the protective capacity of each group of determinants, both separately and in combination.

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