A *Theileria annulata* DNA Binding Protein Localized to the Host Cell Nucleus Alters the Phenotype of a Bovine Macrophage Cell Line

Brian R. Shiels,† Sue McKellar, Frank Katzer, Kim Lyons, Jane Kinnaird, Chris Ward, Jonathan M. Wastling, and David Swan

Department of Veterinary Parasitology, Institute of Comparative Medicine, University of Glasgow, Glasgow G61 1QH, and Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom

Received 18 August 2003/Accepted 25 November 2003

Intracellular parasites within the phylum Apicomplexa (e.g., *Babesia, Eimeria, Plasmodium, Theileria*, and *Toxoplasma* spp.) include some of the most important pathogens of humans and domesticated animals. To establish and survive, intracellular parasites modulate the host cell environment (42). Evidence that this process involves interfering with the molecular pathways that determine the phenotypes of mammalian cells is accumulating, and apicomplexan parasites have been shown to alter the control of cellular differentiation (29), proliferation (12), and apoptosis (21).

*Theileria annulata* and *Theileria parva* are tick-borne apicomplexans that cause fatal and debilitating diseases of cattle throughout large areas of Africa and Asia. A primary factor in the pathology of these diseases is the generation of neoplasia by rapidly dividing transformed infected leukocytes (6). Host cell division and dedifferentiation are induced by the intracellular multinucleated macroschizont stage (18, 29) and are accompanied by alterations to bovine gene expression (30). These events are dependent on a viable macroschizont, as they can be reversed by the treatment of cells with the theileriacidal drug buparvaquone (22). Interestingly, macroschizont-dependent alterations include molecules that are involved in controlling proliferation or that protect against apoptosis (8, 12, 26). In contrast to what occurs in the macroschizont-infected cell, proliferation of the infected leukocyte slows down and then stops during differentiation to the next parasite life cycle stage, the merozoite stage. An enlargement of the macroschizont accompanies the reduced level of leukocyte division and is followed by the production of multiple uninucleated merozoites (32). Merozoites are then released as the host cell ruptures and invade erythrocytes to form the intracellular piroplasm stages infective for the tick vector (23).

*Theileria* molecules that can modulate the phenotype of the host cell have not been identified, and the mechanism that down-regulates leukocyte division during differentiation to the merozoite is unknown. Parasite polypeptides (TashATs) that have the potential to modulate bovine gene expression have been described previously. The TashAT polypeptides possess domains required for binding to AT-rich DNA (AT hooks) and for transportation to the host cell nucleus (signal peptide plus AT hooks), and experimental evidence for both of these functions has been reported (37, 38). In addition, the down-regulation of TashAT gene expression during parasite differentiation is coincident with the initial reduction of leukocyte proliferation in vitro, and mammalian AT hook-containing proteins are known to be associated with the generation of neoplasia (4). From these results, it can be postulated that following the removal of TashAT expression, or an undefined macroschizont-encoded signal, host cell division slows down and eventually ceases. However, in this process, it is also possible to propose a more complicated mechanism involving parasite molecules that are required to maintain or alter the modulation of the host cell phenotype during differentiation to the merozoite. The present study has characterized a gene encoding a predicted protein with a signal peptide, DNA binding domains, a PEST region, and motifs that allow interaction with regulatory components of the higher eukaryotic cell cycle. We present evidence that the *Theileria* polypeptide is localized to host nuclei at significant levels in cells that are undergoing parasite differentiation and that it has the ability to modulate the phenotype of an immortalized bovine macrophage cell line.
MATERIALS AND METHODS

Cell culture and nuclear extract preparation. The cloned *T. annulata*-infected cell line D7, the uninfected bovine lymphosarcoma line, and its *Theileria*-infected counterpart were maintained in culture at 37°C as previously described (37). The D7 cell line was induced to differentiate to merozoites by being cultured at 41°C (32). Nuclear fractions were obtained by using standard methods (37). Host- and parasite-enriched nuclear pellets were resuspended in 1 ml and 250 μl, respectively, of buffer A (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol) and lysed following the addition of an equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and the DNA was sheared by passage through a 25-gauge needle or extracted by incubation in 300 mM NaCl as previously described (33). The transformed bovine macrophage cell line BoMac was cultured at 37°C as described previously (36).

Cloning and characterization of the *SuAT1* gene. A clone representing a 2,236-bp fragment of *SuAT1* was isolated from a λgt11 expression library representing parasite-enriched genomic DNA isolated from the infected D7 cloned cell line. The library was screened with a radiolabeled double-stranded concatenated CAT1 probe as previously described (38). The λgt11 insert was PCR amplified with primers flanking the cDNA and protein analyses were performed by using the Genetics Computer Group sequence analysis package (11). The 2,236-bp fragment contained a contiguous open reading frame (ORF) of 1,620 bp but lacked the 5′ end of the putative polypeptide. To obtain the missing residues, a cDNA clone was isolated from a ZAP library (Stratagene) representing mRNA isolated from the protozoan T. annulata (Ankara stock). The resulting sequence of the 5′ end of the cDNA showed high identity to the genomic clone (98% in a 387-bp overlap) and contained the putative methionine start codon plus untranslated sequence. *T. annulata* can display significant sequence diversity, and the Ankara stock is known to contain a number of parasite genotypes (15); therefore, a 322-bp fragment spanning the 5′ end of the ORF was PCR amplified from cell line D7 genomic DNA by using Pfu Turbo DNA polymerase (Stratagene). Sequence analysis revealed two nucleotide alterations relative to the cDNA sequence, one of which resulted in the substitution of a P for an A at residue 7 of the putative polypeptide. Two possible PCR errors were also detected in the ORF of the genomic contig; these errors were corrected after a comparison with the D7 genomic DNA. Sequence identity searches were performed by using BLAST homology information (NCBI) database with the Mascot search engine (Matrix Science, London, United Kingdom).

Establishment and characterization of a *SuAT1*-transfected BoMac cell line. Work on the TashAT cluster genes has shown that increased expression in BoMac cells was obtained by use of a modified vector (p5S11) that was generated by removing the cytomelagovirus immediate early promoter region of the pIREShyg2 vector (Clontech) and replacing it with cytomelagovirus immediate early enhancer, chicken β-actin promoter, and synthetic intron of the pCX-EGFP vector (39). This vector was used to ensure the expression of *SuAT1* at high levels since it can produce a single RNA transcript representing the inserted gene of interest linked to an internal ribosomal entry sequence and Hg mRNA. The *SuAT1* coding region (amino acids 32 to 558) minus the predicted signal peptide was PCR amplified with Pfu Turbo DNA polymerase and the primers SATE1 (5′-GGCGAGTCTGAGCCTACATGAGAATGTCTGCTCATTG-3′), containing an Nhel site and the Kozak consensus sequence, and SATE2 (5′-ACTGTGATCTAGACGTCTTCCTCGTCCGAGTAGTTGTCAGTT-3′), containing a BamHI site. The PCR fragment was then digested with Nhel and BamHI, gel purified, and ligated into the Nhel and BamHI sites of p5S11. The insertion of the resulting p5S11-SuAT1 construct was confirmed for fidelity by sequencing.

Stable transfection of BoMac cells was carried out by using SuperFect transfection reagent (QiAGEN) according to the manufacturer’s protocol. Hygromycin (200 μg/ml) was then added to the cultures 48 h later to select for stable transfectants. A confluent culture was obtained for cells transfected with the control vector p5S11 within 18 days. In contrast, the p5S11-SuAT1 cells took longer to establish a permanent line and appeared to be derived from a single cluster of cells. All nontransfected control cultures failed to establish a permanent line. To estimate cell numbers, test and control cultures were plated at a density of 2×10⁶ cells per flask (four replicates) and incubated for 5 to 6 days in the presence or absence of hygromycin. Cells were harvested and resuspended in RPMI medium, and viable cells were counted by use of trypan blue exclusion and a hemocytometer. The differences between means were tested by t distribution analysis. Culture morphology was assessed by paraffinoldehyde fixation and phase-contrast microscopy. For drug-free cultures, experiments were performed at least 4 weeks after removal of hygromycin.

Nanospray tandem mass spectrometry and database searching. Bands of interest were excised from Coomassie blue-stained SDS-PAGE gels and subjected to in-gel trypsin (Promega, Madison, Wis.) digestion overnight at 37°C by using a previously described method (19). Digestion products were desalted by using C₃, C₁₂ ZipTips (Millipore, Billerica, Mass.) prior to analysis with the QSTAR Pulsar Pulsar tandem mass spectrometer running in the positive ion mode and the Analyst QS software package (Applied Biosystems, Foster City, Calif.) in conjunction with Proxeon (Odense, Denmark) nanospray capillaries. Data were collected from 400- to 1,200-Da proteins, and peaks were selected for mass spectrometry-mass spectrometry by using an IDA filter. The mass spectrometry-mass spectrometry data were searched against the National Center for Biotechnology Information (NCBI) database with the Mascot search engine (Matrix Science, London, United Kingdom).

Nucleotide sequence accession number. The sequence reported here has been submitted to the DDJB/EMBL/GenBank databases under accession number AJ105117.

RESULTS

Isolation and sequence analysis of the *SuAT1* gene. Previous studies indicated that the decision to undergo parasite differentiation involves a quantitative increase in DNA binding factors that control merozoite gene expression (35), and a parasite nucleotide motif (CAT1) that specifically binds *Theileria* nuclear factors has been identified (33). To characterize polypeptides with the potential to bind to this motif, a genomic

Coomassie blue staining of these sample gels, adjustments were made to the sample volumes to give equal track loadings of protein. For the nuclear protein concentration was estimated by the Bradford assay (5a), and equal concentrations (30 μg) were loaded onto SDS-PAGE gels. For the following anti-actin and anti-α-actinin (sc-7453, 1,200), anti-NF-xB p65 (sc-7151, 1,500), anti-Oct-2 (sc-223, 1,200), anti-pan cytokeratin (sc-15367, 1,200), and antivimentin (sc-7558, 1,200) (all from Santa Cruz); and anti-p53 (NCL-p53-CMI, 1,500; Novocastra Laboratories, Ltd.). Anti-SuAT1 was used at a 1:400, anti-*T. annulata* ribonucleotide reductase was used at a 1:500, and MAB 5E1 was used as an unlinked culture supernatant.

Establishment and characterization of a *SuAT1*-transfected BoMac cell line. Work on the TashAT cluster genes has shown that increased expression in BoMac cells was obtained by use of a modified vector (p5S11) that was generated by removing the cytomegalovirus immediate early promoter region of the pIREShyg2 vector (Clontech) and replacing it with cytomegalovirus immediate early enhancer, chicken β-actin promoter, and synthetic intron of the pCX-EGFP vector (39). This vector was used to ensure the expression of *SuAT1* at high levels since it can produce a single RNA transcript representing the inserted gene of interest linked to an internal ribosomal entry sequence and Hg mRNA. The *SuAT1* coding region (amino acids 32 to 558) minus the predicted signal peptide was PCR amplified with Pfu Turbo DNA polymerase and the primers SATE1 (5′-GGCGAGTCTGAGCCTACATGAGAATGTCTGCTCATTG-3′), containing an Nhel site and the Kozak consensus sequence, and SATE2 (5′-ACTGTGATCTAGACGTCTTCCTCGTCCGAGTAGTTGTCAGTT-3′), containing a BamHI site. The PCR fragment was then digested with Nhel and BamHI, gel purified, and ligated into the Nhel and BamHI sites of p5S11. The insertion of the resulting p5S11-SuAT1 construct was confirmed for fidelity by sequencing.

Stable transfection of BoMac cells was carried out by using SuperFect transfection reagent (QiAGEN) according to the manufacturer’s protocol. Hygromycin (200 μg/ml) was then added to the cultures 48 h later to select for stable transfectants. A confluent culture was obtained for cells transfected with the control vector p5S11 within 18 days. In contrast, the p5S11-SuAT1 cells took longer to establish a permanent line and appeared to be derived from a single cluster of cells. All nontransfected control cultures failed to establish a permanent line. To estimate cell numbers, test and control cultures were plated at a density of 2×10⁶ cells per flask (four replicates) and incubated for 5 to 6 days in the presence or absence of hygromycin. Cells were harvested and resuspended in RPMI medium, and viable cells were counted by use of trypan blue exclusion and a hemocytometer. The differences between means were tested by t distribution analysis. Culture morphology was assessed by paraffinoldehyde fixation and phase-contrast microscopy. For drug-free cultures, experiments were performed at least 4 weeks after removal of hygromycin.

Nanospray tandem mass spectrometry and database searching. Bands of interest were excised from Coomassie blue-stained SDS-PAGE gels and subjected to in-gel trypsin (Promega, Madison, Wis.) digestion overnight at 37°C by using a previously described method (19). Digestion products were desalted by using C₃, C₁₂ ZipTips (Millipore, Billerica, Mass.) prior to analysis with the QSTAR Pulsar Pulsar tandem mass spectrometer running in the positive ion mode and the Analyst QS software package (Applied Biosystems, Foster City, Calif.) in conjunction with Proxeon (Odense, Denmark) nanospray capillaries. Data were collected from 400- to 1,200-Da proteins, and peaks were selected for mass spectrometry-mass spectrometry by using an IDA filter. The mass spectrometry-mass spectrometry data were searched against the National Center for Biotechnology Information (NCBI) database with the Mascot search engine (Matrix Science, London, United Kingdom).

Nucleotide sequence accession number. The sequence reported here has been submitted to the DDJB/EMBL/GenBank databases under accession number AJ105117.

RESULTS

Isolation and sequence analysis of the *SuAT1* gene. Previous studies indicated that the decision to undergo parasite differentiation involves a quantitative increase in DNA binding factors that control merozoite gene expression (35), and a parasite nucleotide motif (CAT1) that specifically binds *Theileria* nuclear factors has been identified (33). To characterize polypeptides with the potential to bind to this motif, a genomic
λgt11 expression library was screened with a concatenated double-stranded CAT1 probe. The screen resulted in the isolation of a single λgt11 clone (SuAT1) that showed significant binding to the DNA probe (Fig. 1A). Southern blotting was performed on DNA derived from an infected cloned cell line and genomic bovine DNA by using an EcoRI fragment of the SuAT1 gene as a probe. A major 6.5-kb EcoRI fragment was specifically detected in parasite-derived DNA, and, as additional bands were also identified under both reduced- and high-stringency conditions (Fig. 1B), it was concluded that additional genes related to SuAT1 are carried within the T. annulata genome. This result has been confirmed by the identification of a further two genes closely related to SuAT1 within the T. annulata genomic database (http://www.sanger.ac.uk/Projects/T_annulata/blast_server.shtml). No signal was obtained with bovine genomic DNA. Additional bands were also obtained by Northern blot analysis (Fig. 1C), as the SuAT1 probe detected two mRNA species of 2.2 and 4.4 kb. The 2.2-kb species was detected in RNA derived from macroschizont-infected cells (day 0) and in RNA isolated from differentiating parasites (day 7), while the 4.0-kb species was detected only in RNA isolated from a differentiating culture.

The sequence of the complete ORF of SuAT1 was found to be uninterrupted by introns and encoded 558 amino acids (Fig. 2A). A comparison of the SuAT1 sequence with the NCBI protein databases showed relatedness to the TashAT and Tash1 polypeptides of T. annulata (38, 40). Of particular interest was the conservation of a number of previously identified motifs that predict a DNA binding function and/or nuclear localization. Thus, the SuAT1 sequence contained an AT hook motif (Fig. 2B) with an RGRP core that was identical to AT hook domains previously identified for the TashAT proteins and highly related to the AT hooks of the HMGIY polypeptide (38). In addition, the regions that flank the consensus AT hook and align with the first and third AT hooks of TashAT2 and TashAT1-3 are also rich in basic amino acid residues and may bind DNA. The SuAT1 sequence also encoded three potential nuclear localization signals, one of which is a bipartite nuclear localization signal encompassing the AT hook. A further potential bipartite nuclear localization signal was identified at positions 151 to 169. This motif has been found to be part of the DNA binding domain of a number of proteins, including the HMGI box of ROX1 (5), and was predicted to bind to DNA (10).

Other motifs with predicted functions were identified in the SuAT1 sequence. These motifs included an N-terminal signal peptide with a predicted cleavage site between residues 23 and 24. Interestingly, an RXL cyclin interaction site (16) and a (ST)PXR(KRX) CDK phosphorylation site (24) were also identified and shown to be conserved across the SuAT1 and TashAT genes (Fig. 2C and D). A sequence domain identified as a small repeated region conserved between Tash1 and the TashATs was also predicted for SuAT1 (Fig. 2E) and was found to be part of two potential PEST sequences located at positions 343 to 368 and 491 to 519 (PEST-FIND scores of 14.34 and 8.31, respectively). PEST motifs act as a signal for proteolytic degradation and are found in a number of functionally important cellular polypeptides (28).

SuAT1 is localized to the host cell nucleus during parasite differentiation. The possession of a predicted signal sequence and nuclear localization allow the postulation that the SuAT1-encoded polypeptide is transported to the host cell nucleus. To test this theory, immunofluorescence analysis was performed on Theileria-infected cells. The results showed that antiserum against the recombinant SuAT1 polypeptide clearly detected both the parasite and host nuclei of macroschizont-infected
cells, compared to the reactivity obtained with preimmune serum and uninfected BL20 bovine lymphoblastoid cells (Fig. 3, panels 1 to 3). Moreover, reactivity against the host cell nuclei was detected at elevated levels in cells of cultures placed at 41°C for both 4 and 7 days (Fig. 3, panels 4 to 6) relative to an antibody against the Oct-2 transcription factor that showed constitutive levels of reactivity at these time points (Fig. 3, panels 7 to 9). As the day 7 time point has been shown to be associated with a cessation of host cell proliferation (32) and the production of merozoites, it was concluded that the SuAT1 polypeptide is present in the host nuclei of cells undergoing merogony. This result was confirmed by triple-labeling experiments carried out with anti-SuAT1, a MAb specific for the merozoite surface antigen Tams1, and DAPI (4′,6′-diamidino-2-phenylindole) labeling of host and parasite nuclei. Strong host nuclear reactivity was clearly detected in cells containing parasites that were undergoing merozoite nuclear production and expressing a high level of the major merozoite surface antigen (Fig. 4A, panels 1 to 4). Double labeling with anti-SuAT1 and DAPI of nondifferentiating macroschizont-infected cells was also performed and showed that occasional cells which possessed a level of host nuclear reactivity that was higher than that of the majority of the culture tended to contain enlarged parasites (Fig. 4A, panels 5 and 6).

Immunoblotting experiments (Fig. 4B) with the SuAT1 antibody specifically detected a molecule in total, host nuclear, and parasite-enriched nuclear extracts of infected cells that was virtually identical in mass (100 kDa) to the polypeptide detected in nuclear extracts of mammalian BoMac cells stably transfected with a SuAT1 expression construct (Fig. 5D). The level of the 100-kDa SuAT1 polypeptide was enriched in host nuclear extracts derived from macroschizont-infected (37°C) cultures and cells undergoing differentiation towards the merozoite. This pattern of localization was in contrast to those of two larger polypeptides that were detected at reduced levels in the host nuclear extract of a differentiating culture, which was virtually identical in mass (100 kDa) to the polypeptide detected in nuclear extracts of mammalian BoMac cells stably transfected with a SuAT1 expression construct (Fig. 5D). The level of the 100-kDa SuAT1 polypeptide was enriched in host nuclear extracts derived from macroschizont-infected (37°C) cultures and cells undergoing differentiation towards the merozoite. This pattern of localization was in contrast to those of two larger polypeptides that were detected at reduced levels in the host nuclear extract of a differentiating culture, which from
their estimated masses (150 and 180 kDa) may represent cross-recognition of TashAT2 and TashAT3. A blot was also probed with an antiserum raised against the large subunit of ribonucleotide reductase (*T. annulata*) that specifically reacts to the parasite nucleus by immunofluorescence (39), and only a limited level of contamination of the host nuclear fraction was detected. To verify the immunofluorescent test result that indicated elevated SuAT1 levels in the host nuclei of cells undergoing differentiation to merozoites, immunoblotting was performed on host nuclear extracts derived from day 0, 4, and 6 cultures. Relative to those of the Oct-2 nuclear antigen, the levels of SuAT1 detected were greater in the extracts derived from the day 4 and 6 cultures than those detected in the day 0 extract (Fig. 4C). A minor band that migrated just above the main 100-kDa band was also detected. The lack of this doublet in transfected cells (Fig. 5D) and the detection of a band in an identical position with antisera against another SuAT-related recombinant polypeptide (B. R. Shiels and S. McKellar, unpublished data) indicate that the presence of the upper band is likely to be due to the recognition of a SuAT1-related polypeptide. Based on the immunofluorescence and immunoblot analyses, we concluded that the SuAT1 polypeptide is present at significant levels in the host nuclei of macroschizont-infected cells and cells that are undergoing differentiation into merozoites.

**SuAT1 modulates the phenotype of BoMac cells.** The experimental evidence outlined above, combined with the identification of sequence domains that predict modulation of gene expression and possible interaction with regulatory complexes that control the cell cycle, led us to postulate that SuAT1 may be required for parasite-mediated control of the host cell phenotype. In *vivo*, *T. annulata* is thought to readily infect cells of
Therefore, to examine the effect of SuAT1 on this cell type and to determine if the sequence encodes functional mammalian nuclear localization signals, the transformed bovine macrophage cell line BoMac (36) was transfected with the plsl1-SuAT1 construct. A single cell line stably transfected with SuAT1 was obtained but took longer to become established relative to cell lines transfected with the expression vector control (plsl1). When cell growth was analyzed (Fig. 5A), SuAT1-transfected cultures incubated in the presence of hygromycin showed a significant reduction (1.8-fold) in the number of cells relative to those of the control cultures. One explanation for these results was that the SuAT1-transfected cells expressed a lower level of resistance to hygromycin than the control cells. This possibility was supported by the increased level of growth relative to that of the control cells following the removal of hygromycin (Fig. 5A). Therefore, to control for a possible detrimental effect of hygromycin on the SuAT1-transfected cells, all subsequent analyses included SuAT1-BoMac cells that were cultured in the absence of hygromycin for 4 weeks or more.

An examination of cell morphology showed that the expression of SuAT1 had a marked effect on BoMac cells. Relative to the plsl1-BoMac constructs, the SuAT1-BoMac cells appeared to be more spread out and significantly larger and possessed larger nuclei (Fig. 5B). These general changes in morphology were specific to the SuAT1 polypeptide, immunofluorescence and immunoblotting were performed with the anti-SuAT1 serum. The results showed that a 100-kDa polypeptide was specifically localized to the nuclei of the SuAT1-transfected cells cultured in the absence or presence of hygromycin (Fig. 5C and D).

SuAT1 and T. annulata modulate the expression pattern of cytoskeletal proteins. To investigate whether the SuAT1-associated alteration of BoMac morphology was linked to changes in the profile of cellular polypeptides, protein extracts of SuAT1- and plsl1-transfected BoMac cells were separated by SDS-PAGE. Initial analysis was performed with total extracts, and alterations were observed in major polypeptides of 53 and 70 kDa, suggesting a potential link to cytoskeletal proteins. Further studies will be required to elucidate the specific changes in expression and their functional implications in the altered morphology of SuAT1-transfected BoMac cells.
41 kDa (data not shown). Subsequent analysis of isolated nuclei extracted with SDS sample buffer confirmed and clarified these alterations and identified modulations of other polypeptides. These alterations are shown in Fig. 6A and were manifest in SuAT1-BoMac cells grown in both the presence and the absence of hygromycin as a reduction in a band at approximately 100 kDa, an increase in a band at 55 kDa, a major reduction in bands at 53 and 41 kDa, and a possible reduction in a band at 43 kDa. As the major alterations at 53 and 41 kDa were not detected in nuclear fractions extracted by 300 mM NaCl (data not shown), it was postulated that the expression of SuAT1 modulates the profile of major polypeptides that may be components of cytoskeletal intermediate filaments (1). This postulation was tested by excising gel slices containing the representative bands, fragmenting the proteins with trypsin, and sequencing the resulting peptides by nanospray tandem mass spectrometry. The resulting peptide sequences were then used to search the NCBI database for identity. The most significant matches for each band (Table 1) were as follows: band 1, actinin; band 2, vimentin; band 3, cytokeratin 8; band 4, actin; and band 5, cytokeratin 19. These results provide evidence that SuAT1 modulates the expression profile of cytoskeletal polypeptides in BoMac cells. Confirmation of these alterations was obtained by using commercial antisera specific for cytoskeletal polypeptides to probe immunoblots of total cellular and nuclear fraction protein extracts. As shown in Fig. 6B and C, the immunoblots provided further evidence that alterations to the nuclear profile of actinin, vimentin, and cytokeratin were associated with SuAT1-transfected BoMac cells, while no significant differences were observed with the antiactin antibody. In addition, it appeared that levels of tubulin associated with the nuclear fractions were increased in SuAT1-BoMac cells relative to that of actin. However, the only alteration detected with the total protein extracts was the marked reduction of cytokeratin levels (Fig. 6B).

The results described above show that a *Theileria* gene lo-
calized to the host nucleus is associated with an alteration of the BoMac cell phenotype and the expression profile of structural polypeptides. To test whether changes to the cytoskeleton are associated with Theileria infection, we repeatedly attempted to infect the BoMac cells with T. annulata sporozoites but did not succeed, and we have been unable to transfect the uninfected BL20 cell line with SuAT1 (data not shown). The reason for this finding is unclear, but as an invasion of sporozoites appeared to have taken place, it may be that prior transformation of the macrophage is refractory to macroschizont establishment. Therefore, we analyzed the profile of cytoskeletal polypeptides in total extracts of the infected D7 cell line after treatment with the theileriacidal drug buparvaquone and during a time course of differentiation into merozoites (Fig. 6D). No reactivity was obtained with the antivimentin antibody, and no alteration to the level of actinin was detected (data not shown). Interestingly, the level of a 53-kDa polypeptide detected by the anticytokeratin antibody showed a significant increase in extracts from buparvaquone-treated cells compared to that in untreated extracts. This increase was not

**FIG. 6.** SDS-PAGE and immunoblot analyses of SuAT1-transfected and Theileria-infected cell lines. (A) SDS-PAGE analysis of nuclear extracts derived from BoMac cells transfected with pls11 or pls11-SuAT1 (SuAT) constructs cultured in the presence (+) or absence (−) of hygromycin; (B) total (T) and nuclear (N) extracts of BoMac cells transfected with pls11 or pls11-SuAT1 (SuAT) constructs cultured in the presence (+) or absence (−) of hygromycin; (C) normal BoMac nuclear extract (BMc) and multiple nuclear extracts of BoMac cells transfected with pls11 or pls11-SuAT1 (SuAT) constructs cultured in the presence (+) or absence (−) of hygromycin; (D) total extracts of infected D7 cells treated with buparvaquone for 72 h (Bpq); extracts from untreated D7 cells cultured at 37°C (day 0) and 41°C for 2, 4, and 6 days; and extracts of uninfected (BL) and infected (TBL) BL20 cells. Immunoblots were probed with antibody against the designated polypeptides actinin, actin, tubulin, pan-cytokeratin, vimentin, NF-κB-p65, and Tams1.

---

**TABLE 1.** Tryptic peptides of BoMac cytoskeletal proteins sequenced by mass spectrometry

| Band | Most significant match | NCBI accession no. | Score \(^a\) | Peptides matched |
|------|------------------------|--------------------|--------------|-----------------|
| 1    | Alpha actinin          | NP_068695          | 1,478        | ETTDTDTADOVIASFK plus 25 others |
| 2    | Vimentin               | NP_776349          | 647          | DGQVINETSOHHDPL plus 19 others |
| 3    | Cytokeratin 8          | P05786             | 505          | LEGLTDINFNY plus 14 others |
| 4    | Actin                  | ATB0G              | 792          | LCYVLDFEQEMATAASSSLEK plus 16 others |
| 5    | Cytokeratin 19         | P08728             | 700          | QSSSTTSSFGGMGGM plus 20 others |

\(^a\) The score is \(-10 \times \log P\), where \(P\) is the probability that the observed match is a random event. Individual ions scores greater than 49 indicate identity or extensive homology (\(P < 0.05\)).
detected with either the tubulin or the actin antibodies. We also tested the levels of cytokeratin in extracts of uninfected and *Theileria*-infected BL20 cells. The results indicate that infection by *Theileria* is associated with a reduction in cytokeratin levels. Analysis of the differentiation time course did not show major differences in the level of cytokeratin relative to that of macroschizont-infected cells at day 0. In contrast, the levels of actin showed a significant reduction relative to those of tubulin and NF-κB as early as day 2, and this reduction continued through to day 4, while both actin and tubulin polypeptides appeared to show reduced levels at day 6 relative to that of p65 (Fig. 6D). There was no reactivity with the antiactin antibody against a parasite-enriched piroplasm extract (data not shown). An elevation in the level of the major merozoite antigen, Tams1, demonstrated that the parasite had undergone differentiation to the merozoite.

**DISCUSSION**

Altering the host cell environment is likely to be essential for the establishment, survival, and reproduction of intracellular parasites, in general (42). For some parasites, this process will involve the subjugation of the molecular pathways that control fundamental cellular processes such as differentiation, apoptosis, and division. *Theileria* parasites provide a good model to study these events because the parasite dedifferentiates the host cell, alters the profile of gene expression, and modulates the pathways that regulate leukocyte proliferation and apoptosis. Control over the leukocyte has largely been attributed to the macroschizont stage of the parasite. From the data generated in this study, however, it is feasible that parasite-mediated control of the host cell is actively maintained as the macroschizont undergoes stage differentiation to the merozoite.

SuAT1 was isolated by the ability of the encoded polypeptide to bind DNA, and two DNA binding motifs were identified in the predicted amino acid sequence, one of which showed high identity to AT hook motifs that have been found in a wide range of eukaryotic DNA binding proteins (3), including the TashAT factors of *T. annulata* (37, 38). This finding may explain the binding of SuAT1 to the CAT1 concatenated probe, as CAT1 has an AT-rich motif that also binds to an AT hook bearing a fragment of TashAT1 (38). Although SuAT1, like the TashAT polypeptides, is expressed in the macroschizont-infected cell, it is unlikely, for several reasons, to perform an identical functional role. First, while the conserved AT hook motif suggests that SuAT1 can bind to the narrow minor groove of AT-rich DNA and influence transcriptional regulation of its target genes, the divergence from the first and third AT hooks of TashAT2 and TashAT1-3 and the possession of the second predicted DNA binding domain indicates that SuAT1 may recognize a different subset of target genes. Second, the RNA and polypeptide expression profiles of SuAT1 are distinct from those of the TashATs; during the differentiation process, TashAT2 and TashAT3 are down-regulated at the same time (between days 2 and 4) that host cell division begins to subside, whereas the level of SuAT1 in the leukocyte nucleus was detected at elevated levels at the day 4 time point, and SuAT1 expression was clearly maintained in cells with parasites undergoing merozoite production. It appears, therefore, that there is a requirement for SuAT1 in the host nuclei of infected cells at time points associated with the inhibition of host cell division. Thus, it is unlikely that SuAT1 is involved in the stimulation of host cell proliferation, although it is possible that its function is altered during differentiation by a changeover of partner binding proteins or a reduction in the level of macroschizont-specific regulatory proteins.

Transfection of the transformed BoMac cell line was carried out, which confirmed that the SuAT1 polypeptide has structural information for localization to the host cell nucleus. Under constant hygromycin selection pressure, the level of growth of the SuAT1-transfected cells was significantly reduced. While this result was most likely due to a reduced level of resistance of these cells to hygromycin, it is also possible that SuAT1 itself may alter cell viability. Indeed, we have found that the expression of SuAT1 in transfected COS7 cells in the absence of drug limits culture growth (Shiels and McKellar, unpublished data), and as SuAT1 possesses both cyclin docking and CDK phosphorylation motifs, it might interact with regulatory complexes of the cell division cycle. Experiments with an inducible expression system being used to test the influence of SuAT1 on cell division and/or apoptosis are in progress.

As shown in Fig. 5, the expression of SuAT1 was associated with altered morphology of the BoMac cells. Transfected cells were also shown to have an altered expression profile of cytoskeletal polypeptides, and based on their role in determining cell structure (1), it is possible that these events are linked. How these changes are brought about requires clarification, but as the changes to actin, tubulin, and vimentin were not detected in total extracts, they are unlikely to result from direct changes to the control of expression of cytoskeletal genes and may be due to a reorganization of the cytoskeleton. In contrast, levels of cytokeratin polypeptides appear to be repressed following transfection with SuAT1, and analyses of extracts derived from buparvaquone-treated macroschizont-infected cells indicated that parasite infection results in lower levels of a 53-kDa cytokeratin polypeptide (Fig. 6D). The low level of expression was maintained during parasite differentiation, and we also detected a lower level of the 53-kDa polypeptide in macroschizont-infected cells compared to uninfected BL20 lymphoblastoid cells. Thus, it appears that both SuAT1 and the parasite are associated with the repression of cytokeratin gene expression. This is a surprising result, as cytokeratins are thought to be intermediate filaments of epithelial cells and are not normally associated with cells of lymphoid or myeloid lineage (1). However, as the aberrant expression of cytokeratin genes has been detected in transformed cells of nonepithelial origin (20), it can be postulated that this also occurs following transformation by *T. annulata*. This may in turn be detrimental to parasite establishment and may require repression by the macroschizont in the infected cell. Recent work has shown that the expression of cytokeratin polypeptides can both induce and inhibit cellular proliferation (27).

Mammalian AT hook polypeptide control gene expression (41), and it has been demonstrated that they are involved in the determination of cell fate by the modulation of differentiation and division of myeloid cells (4, 7). *Theileria* AT hook polypeptides may perform similar functions in infected cells, but this has yet to be determined. Nevertheless, SuAT1 does have the potential to alter the phenotype of mammalian cells, and it is conceivable that this function provides a selective advantage.
for the parasite. For example, *Theileria* infection of leukocytes alters the morphology of these cells, and it is known that the macronchizont interacts with components of the host cell cytoskeleton (31). Differentiation to the merozoite is also associated with significant changes to the leukocyte phenotype; during parasite differentiation, the leukocyte stops dividing, enlarges, becomes filled with the differentiating parasite, and is eventually destroyed (32). These events may require active control via parasite molecules transported to the host cell compartment. It may also be relevant that the level of cellular control via parasite molecules transported to the host cell eventually destroyed (32). These events may require active control.

Theileria cruzi alters the morphology of these cells, and it is known that the trans-sialidase of *Theileria parva* and it is known that the trans-sialidase of *Theileria parva* and *Theileria annulata* (31). Differentiation to the merozoite is also associated with the cytokines ciliary neurotrophic factor and leukemia inhibitory factor to prevent apoptosis of neuronal cells. Mol. Biol. Cell 11:1487–1498.

Cokol, M., R. Nair, and B. Rost. 2000. Finding nuclear localization signals. EMBO Rep. 1:411–415.

Doverrex, J., P. Haerbert, and O. Smithies. 1984. A comprehensive set of sequence alignment programs for the VAX. Nucleic Acids Res. 12:387–395.

Dubbelaere, D., and V. Huessler. 1999. Transformation of leukocytes by *Theileria parva* and *T. annulata*. Annu. Rev. Microbiol. 53:1–42.

Gardner, M. J., N. Hall, E. Fung, O. White, M. Berriman, R. W. Hyman, J. M. Carlton, A. Pain, K. E. Nelson, S. Bowman, I. T. Paulsen, K. James, I. A. Ades, K. Rutherford, R. White, S. Baldwin, A. Craig, S. Kyes, M. S. Chan, Y. Nene, S. J. Shallom, B. Suh, J. Peterson, S. Angiuoli, M. Pertea, J. Allen, J. Selengut, D. Haft, M. W. Mather, A. B. Vaidya, D. M. Martin, A. H. Fairlamb, M. J. Fraunholz, D. S. Roos, S. A. Ralph, G. I. McFadden, L. M. Cummings, G. M. Subramanian, C. Mungall, J. C. Venter, D. J. Carucci, S. L. Hoffman, C. Newbold, R. W. Davis, C. M. Fraser, and B. Barrett. 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419:496–511.

Glass, E. J., E. A. Innes, R. L. Spooner, and C. G. Brown. 1989. Infection of bovine monocyte macrophage populations with *Theileria annulata* and *Theileria parva*. Vet. Immunol. Immunopathol. 22:355–368.

Gubbels, M. J., F. Katzer, G. Hide, F. Jongejan, and B. R. Shieles. 2000. Generation of a mosaic pattern of diversity in the major merozoite-piroplasm surface antigen of *Theileria annulata*. Mol. Biochem. Parasitol. 110:23–32.

Haft, C., D. M. Nelson, X. Ye, K. Baker, J. A. DeCaprio, S. Seelholzer, M. Lipinski, and P. D. Adams. 2001. HIRA, the human homologue of yeast Hir1p and Hir2p, is a novel cyclin-cdk2 substrate whose expression blocks S-phase progression. Mol. Cell. Biol. 21:1845–1865.

Huang, S., and D. E. Ingber. 2000. Shape dependent control of cell growth, differentiation, and apoptosis: switching between attractors in cell regulatory networks. Exp. Cell Res. 261:91–103.

Hughler, L. 1965. Cultivation of three species of *Theileria* in lymphoid cells in vitro. J. Protozool. 12:649–655.

Kinter, M., and N. E. Sherman. 2000. Protein sequencing and identification using tandem mass spectrometry. Wiley Interscience, New York, N.Y.

Knapp, A. C., and W. W. Franke. 1993. Evidence for the induction of casein kinase II in bovine lymphocytes by the intracellular protozoan parasite *Theileria parva*. EMBO J. 12:1621–1631.

Luder, C. G. K., U. Gross, and M. F. Lopes. 2001. Intracellular protozoan parasites and apoptosis: diverse strategies to modulate parasite-host interactions. Trends Parasitol. 17:480–486.

McHardy, N., L. S. Wekesa, A. T. Hudson, and A. W. Randall. 1985. Anti-theilerial activity of BW720C (buparvaquone): a comparison with parvaquone. Vet. Res. Vet. Sci. 39:29–33.

Melhorn, H., and E. Schein. 1984. The piroplasms: life cycle and sexual cycles. Adv. Parasitol. 23:37–103.

Moreno, S., and P. Nurse. 1990. Substrates for p34^cdk2: in vivo veritas? Cell 67:459–551.

Nielsen, H., J. Engelbrecht, S. Brunak, and G. Von Heijne. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10:1–6.

Ole-MoiYoi, O. K., W. C. Brown, K. P. Lams, A. Nayer, T. Tsukamoto, and Y. Minabe. 2000. Protein sequencing and identification using tandem mass spectrometry. Wiley Interscience, New York, N.Y.

Paramio, J. M., M. L. Casanova, C. Segrelles, S. Mittnacht, E. B. Lane, and S. Mittnacht. 1997. Macrophage- parasite relationship in theileriosis. Reversible phenotypic and functional dedifferentiation of macrophages infected with *Theileria annulata*. Mol. Biochem. Parasitol. 86:459–465.

Sager, H., C. Bronschweiler, and T. W. Junge. 1998. Interferon production by *Theileria annulata*-transformed cell lines is restricted to the beta family. Parasite Immunol. 20:175–182.

Shaw, M. K. 1997. The same but different: the biology of *Theileria* sporozoite entry into bovine cells. Int. J. Parasitol. 27:457–474.

6. Brown, C. G. D., A. G. Hunter, and A. G. Lucksin. 1990. Diseases caused by protozoa, p. 161–226. In M. H. Sewell and D. W. Brocklesby (ed.), *Handbook on animal diseases in the tropics*, 4th ed. Bailliere Tindall, London, United Kingdom.

7. Caslini, C., A. Shiliatifard, L. Yang, and J. L. Hess. 2000. The amino terminus of the mixed lineage leukemia protein (MLL) promotes cell cycle arrest by interfering with cdk2-dependent differentiation. Proc. Natl. Acad. Sci. USA 97:2797–2802.

8. Chaussepie, M., D. Lallemand, M. Moreau, R. Adamson, R. Hall, and G. Langsley. 1998. Upregulation of Jun and Fos family members and permanent JNK activity lead to constitutive AP-1 activation in *Theilera*-transformed leukocytes. Mol. Biochem. Parasitol. 94:215–226.

9. Chuenkova, M. V., and M. A. Pereira. 2000. A trypanosomal protein synergizes with the cytokines ciliary neurotrophic factor and leukemia inhibitory factor to prevent apoptosis of neuronal cells. Mol. Biol. Cell 11:1487–1498.

ACKNOWLEDGMENTS

This work was supported by a grant from the Wellcome Trust (059323) and the European Union INCO-DEV program (IC4A-2000-30020). We thank Andy Tait and Collette Britton for discussions and critical reading of the manuscript. We thank Vicky Gillan for Microsoft Excel expertise.

Genomic data were provided by The Institute for Genomic Research (supported by NIH grant number AO5093) and by the Sanger Center (Wellcome Trust). Expressed sequence tag sequences were generated by Washington University (NIH grant number 1R01AI045806-01A1).

REFERENCES

1. Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. 1989. The cytoskeleton, p. 613–680. In M. Robertson (ed.), Molecular biology of the cell, 2nd ed. Garland Publishing, Inc., New York, N.Y.

2. Altshul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

3. Aravind, L., and D. Landsman. 1998. AT-hook motifs identified in a wide variety of DNA-binding proteins. Nucleic Acids Res. 26:4413–4421.

4. Ayton, P. M., and M. L. Cleary. 2001. Molecular mechanisms of leuкоemogenesis mediated by MLL fusion proteins. Oncogene 20:5695–5707.

5. Balasubramanian, B., C. V. Lowry, and R. S. Zitomer. 1993. The Roxl repressor of the Saccharomyces cerevisiae hypoxia genes is a specific DNA-binding protein with a high-mobility-group motif. Mol. Cell. Biol. 13:6071–6078.

5a. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
32. Shiels, B. R., J. Kinnaird, S. McKellar, T. Dickson, L. Ben-Miled, R. Melrose, D. Brown, and A. Tait. 1992. Disruption of synchrony between parasite growth and host cell division is a determinant of differentiation to the merozoite in Theileria annulata. J. Cell Sci. 101:99–107.

33. Shiels, B. R., M. Fox, S. McKellar, J. Kinnaird, and D Swan. 2000. An upstream element of the TamS1 gene is a site of DNA-protein interactions during differentiation to the merozoite in Theileria annulata. J. Cell Sci. 113:2243–2252.

34. Shiels, B. R., A. Smyth, J. Dickson, S. McKellar, L. Tetley, K. Fujisaki, B. Hutchinson, and J. H. Kinaird. 1994. A stoichiometric model of stage differentiation in the protozoan parasite Theileria annulata. Mol. Cell. Differ. 2:101–125.

35. Shiels, B. R. 1999. Should I stay or should I go now: a stochastic model of stage differentiation in Theileria annulata. Parasitol. Today 15:241–245.

36. Stabel, J. R., and T. J. Stabel. 1995. Immortalization and characterization of bovine peritoneal macrophages transfected with SV40 plasmid DNA. Vet. Immunol. Immunopathol. 45:211–220.

37. Swan, D. G., K. Phillips, A. Tait, and B. R. Shiels. 1999. Evidence for localisation of a Theileria parasite AT hook DNA-binding protein to the nucleus of immortalised bovine host cells. Mol. Biochem. Parasitol. 101:117–129.

38. Swan, D. G., R. Stern, S. McKellar, K. Phillips, C. Oura, T. I. Karagenc, L. Studler, and B. R. Shiels. 2001. Characterisation of a cluster of genes encoding Theileria annulata AT hook DNA-binding proteins and evidence for localisation to the host cell nucleus. J. Cell Sci. 114:2747–2754.

39. Swan, D. G., L. Studler, E. Okan, M. Hoffs, J. Katzer, S. McKellar, and B. R. Shiels. 2003. TasHN, a Theileria annulata encoded protein transported to the host nucleus displays an association with attenuation of parasite differentiation. Cell. Microbiol. 5:947–956.

40. Swan, D. G., K. Phillips, S. McKellar, C. Hamilton, and B. R. Shiels. 2001. Temporal co-ordination of macroschizont and merozoite gene expression during stage differentiation of Theileria annulata. Mol. Biochem. Parasitol. 113:233–239.

41. Thanos, D., and T. Maniatis. 1992. The high mobility group protein HMG1(Y) is required for NFκB-dependent virus induction of the human IFN-β gene. Cell 71:777–789.

42. Trager, W. 1986. Living together: the biology of animal parasitism. Plenum Press, New York, N.Y.