Acetyl:succinate CoA-transferase (ASCT) is an enzyme that transfers the CoA moiety of acetyl-CoA to succinate, yielding acetate and succinyl-CoA. ASCT was earlier characterized as an acetate-producing activity in Trypanosomatidae (1). Trypanosomatidae are the earliest branching eukaryotes characterized by excretion of mainly partially oxidized end products like pyruvate, succinate, and acetate. All of the Trypanosomatidae investigated produce acetate to a certain extent during their life cycle (4–7), but the amount of acetate produced differs widely depending on the species and the stage of development.

Trypanosoma brucei, one of the causative agents of African trypanosomiasis, alternates during its life cycle between the bloodstream of its mammalian host and the blood-feeding tsetse insect vector Glossina spp. In the mammalian bloodstream long slender-form parasites proliferate. At the peak of parasitemia, nonproliferative short stumpy cells develop that are prepared to differentiate to procyclic insect stage parasites. The long slender stage depends entirely on glycolysis for energy generation and excretes pyruvate as the main end product of carbohydrate metabolism (8–10). The procyclic stage, on the other hand, can also use amino acids for its energy generation, and its glucose metabolism is completely reorganized, with succinate and acetate as main end products. Recently it was shown that inside the glycosome succinate is produced by a soluble fumarate reductase (11). In the procyclic stage the mitochondrion is directly involved in the degradation of substrates, in contrast to the situation in the long slender bloodstream stage. Pyruvate enters the mitochondrion and is converted by pyruvate dehydrogenase into acetyl-CoA. This acetyl-CoA is not degraded to carbon dioxide via the Krebs cycle but is converted into acetate by ASCT (1, 12). The ASCT reaction concomitantly produces succinyl-CoA, which is recycled to succinate by succinyl-CoA synthetase, an enzyme that also forms succinate and thiamine pyrophosphate (TPP) as main products.

Acetyl:succinate CoA-transferase (ASCT) is an enzyme that transfers the CoA moiety of acetyl-CoA to succinate, yielding acetate and succinyl-CoA. ASCT was earlier characterized as an acetate-producing activity in Trypanosomatidae (1). Trypanosomatidae are the earliest branching eukaryotes characterized by excretion of mainly partially oxidized end products like pyruvate, succinate, and acetate. All of the Trypanosomatidae investigated produce acetate to a certain extent during their life cycle (4–7), but the amount of acetate produced differs widely depending on the species and the stage of development.

Acetylsuccinate CoA-transferase (ASCT) is an enzyme that transfers the CoA moiety of acetyl-CoA to succinate, yielding acetate and succinyl-CoA. ASCT was earlier characterized as an acetate-producing activity in Trypanosomatidae (1). Trypanosomatidae are the earliest branching eukaryotes characterized by excretion of mainly partially oxidized end products like pyruvate, succinate, and acetate. All of the Trypanosomatidae investigated produce acetate to a certain extent during their life cycle (4–7), but the amount of acetate produced differs widely depending on the species and the stage of development.
Acetyl:Succinate CoA-transferase

suum (13, 14). Furthermore, ASCT is also a key enzyme in the metabolism of a wide spectrum of anaerobic protists, including ciliates such as *Nycctotherus ovalis*, chytridiomycete fungi such as *Neocallimastix*, and parabasalids such as *Trichomonas vaginalis* (15–17). In these protists ASCT is located inside their hydrogenosomes, anaerobic energy-generating organelles. Hydrogenosomes are H$_2$-producing, membrane-enclosed organelles related to mitochondria, and they evolved independently in the various protists (18, 19). Sequence comparison of several organelle-specific heat shock proteins or chaperonins and the presence of targeting signals at the N terminus of hydrogenosomal enzymes that resemble mitochondrial import signals showed that hydrogenosomes and mitochondria are evolutionarily related (20–23). They probably originated from the same prokaryotic endosymbiont of the a group of proteobacteria, and it is suggested that hydrogenosomes have evolved by adaptation to anaerobic conditions (24). The exact evolutionary relation between mitochondria and the various types of hydrogenosomes is, however, still debated.

Although the main function in energy generation of both mitochondria and hydrogenosomes is the oxidation of pyruvate and acetyl-CoA, the two types of organelles do not share many similarities. The ASCT/succinyl-CoA synthetase cycle is the only metabolic pathway common to mitochondria and hydrogenosomes (1). For that reason, phylogenetic analysis of ASCTs from different sources could provide a valuable tool to unravel the evolutionary history of the respective energy-generating organelles, the various types of hydrogenosomes, and (ana)erobically functioning mitochondria. Unfortunately, the sequence of not a single ASCT gene is known yet. Here we have identified the gene encoding ASCT of *T. brucei*. Several reverse genetic strategies and subcellular localization studies were used to establish the link between ASCT gene sequence and mitochondrial ASCT enzyme activity. Furthermore, two independent methods of metabolic analysis using $^{13}$C-enriched and $^{14}$C-labeled glucose were applied to wild type and transgenic mutant clones of *T. brucei*. The combined biochemical and genetic approach resulted in the gene identification of *T. brucei* ASCT and also demonstrated that this enzyme is indeed involved in acetate production. However, ASCT is not an essential gene as ASCT is not the only enzyme producing acetate in *T. brucei*.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The sheared DNA clone 7D10 from The Institute for Genomic Research (GenBank™accessions AQ652285 and AQ652287) was obtained from Dr. Najib M. El-Sayed. The complete sequence of that part of chromosome 11 is now available from the TrypDB genome data base (Tb11.02.0290); it is identical with results of sequencing of clone 7D10. A MluI (blunted)/Mcl fragment of the insert of 7D10 was excised and replaced in the reading frame orientation by a blastidicin S resistance *BSD* (25) cassette (Smal/Stu fragment from pHD987 (courtesy of C. Clayton, Heidelberg) containing the PmlI fragment nucleotides 2189–2687 from pCDNA6/V5-His C (Invitrogen) or by a phleomycin resistance cassette targeting a 572-bp fragment of the *T. brucei* hsp60 (Tb11.02.0290); it is identical with results of sequencing of clone H927/4 genomic DNA with the primers 5’-ggcagaattcCAGCTGCCACATACCTCAGG (EcoRI site) and 5’-actggaacctGATCCGGCGCCAACAAAATT (HindIII site) and from a C-terminal fragment excised from plasmid 7D10 digested with EcoRI and BamHI. The two fragments were inserted into a HindIII/BamHI-opened pTSArb (29). Southern blot analysis was done as described before (12) with an ASCT-specific probe (nucleotides 1–314 of the coding region) generated by PCR.

**Trypanosomes, Cell Culture, and Transformations**—The procyclic *T. brucei* cells were cultured at 27 °C in SDM79 medium containing 10% (v/v) heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin, 1 mM glycerol, and 3.5 mg/ml hemin (12, 30) at cell densities between 2 × 10$^6$ ml$^{-1}$ and 1 × 10$^7$ ml$^{-1}$. The EATRO1125 procylic line used in this work was originally isolated from a tsetse experimentally infected with AnTat1.1E bloodstream trypanosomes (31). The AnTat1.1 procylic line was obtained from the same bloodstream AnTat1.1E clone by differentiation in culture (32). The EATRO1125-T7T and the stock 427 derived 29-13 clone 6 procylic cells expressing the T7 RNA polymerase gene and the tetracycline repressor under control of a T7 RNA polymerase promoter are described in Bringaud et al. (28) and in Wirtz et al. (26), respectively. Trypanosomes were infected and selection of drug resistant clones was performed as previously reported (33) with the following modifications: for washing and electroporation, cytomyx was used at 4 °C, and 1 × 10$^7$ cells were electroporated. After electroporation, procylic cells were directly cloned in 30–50% conditioned SDM79 supplemented with 5–10 μg/ml phleomycin (Cayla), 10 μg/ml hygromycin S (Calbiochem), or 15 μg/ml hygromycin B (Roche Applied Science). For generation of clonal colonies the cells were diluted 1:100 and 1:10 in 90% conditioned SDM79 after 24 h and selected in microtiter plates.

**Production of Anti-ASCT Antibodies**—A recombinant fragment corresponding to the first 414 amino acids of ASCT followed by a C-terminal histidine tag was expressed in the Esherichia coli BL21 using the pET16b expression vector (Novagen). After 2 h of induction at 37 °C with 1 mM isopropyl-β-D-thiogalactopyranoside, the cells were harvested by centrifugation, and proteins were purified by nickel affinity chromatography according to the manufacturer’s instructions (Novagen). Antisera were raised in rabbits by 3 injections at 15-day intervals of 200 μg of recombinant purified protein, electro-eluted after separation on SDS-PAGE, and emulsified with complete (first injection) or incomplete Freund’s adjuvant. The antisera was affinity-purified on polyvinylidene difluoride membrane-bound recombinant protein (12–48 kDa) using a N-terminal His$_6$-tagged ASCT2 (Qiagen). Elution with 0.2 M glycine, 1 mM EDTA, pH 2.2, for 10 min was followed by immediate neutralization, stabilization in 200 μg/ml bovine serum albumin, and dialysis against PBS containing 0.02% NaN$_3$. One specific 53-kDa band was detected on the Western blot.

**Western Blot and Immunofluorescence Analyses**—For the Western blot total protein lysates of procyclic form *T. brucei* (1.75 × 10$^8$ ml$^{-1}$) were separated by SDS-PAGE (16%) and transferred to a nitrocellulose membrane (Millipore) (34). The membranes were blocked with PBS, 5% milk powder for 1 h at room temperature. Primary antibodies and secondary antibodies were diluted in PBS, 0.1% Tween 20 with 1% milk powder: rabbit anti-*T. brucei* phosphorylcerate kinase C 1:5,000; rabbit anti-*T. brucei* ASCT 1:500; mouse anti-T. brucei heat shock protein 60 (hsp60) 1:10,000; IRD800 goat anti-mouse IgG (Biomol) 1:5,000; Alexa 680 goat anti-rabbit IgG (Molecular Probes) 1:5,000. Antibody incubations were for 1 h at room temperature. For detection, the membrane was dried and scanned with the Odyssey™ dual wavelength infrared fluorescence scanner (Li-Cor Biosciences, Lincoln, NE).

For immunofluorescence, log phase cells were fixed with formaldehyde as previously described (35). Slides were incubated with 1:100 diluted affinity-purified rabbit anti-ASCT, and the undiluted H85 monoclonal mouse anti-hsp-60p (36) was followed by fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody diluted 1:100 (Bio-Rad) and ALEXA Fluor 588-conjugated goat anti-mouse secondary antibody diluted 1:100 (Molecular Probes). Cells were observed with a Zeiss UV microscope, and images were captured by a MicroMax 1300/YSIS camera (Princeton Instruments) and MetaView software (Universal Imaging Corp.) and assembled in Adobe Photoshop.

**Digitonin Fractonations**—Digitonin fracionations were done as described in Saus et al. (37). Proteinase K digestion was adapted from Sveshnikova et al. (38). The pellet and concentrated supernatant were suspended in 500 μl of trypanosome homogenization buffer without leupeptin (37), and 8 μl of proteinase K (10 mg/ml) were added on ice. The reaction was stopped by adding 6 μl of phenylmethylsulfonyl fluoride (100 μM) in iso-propanol on ice. For thermolysin digestion, the pellet and concentrated supernatant were suspended in 90 and 40 μl, respectively, of 0.1 x Heps/HCl, pH 7.2, 2 mM CaCl$_2$, 0.2 μg/ml leupeptin, 0.6 mM ascorbate, 1 mM dithiothreitol. The pellet and supernatant were incubated with 10 and 5 μl, respectively, of thermolysin (1 mg/ml) for 30 min at 45 °C. The digestion was stopped by adding EDTA on ice.
Acetyl:Succinate CoA-transferase

ASCT Activity Assay—Homogenates of procyclic T. brucei were prepared from 5 × 10^6 cells in 20 mM HEPES buffer, pH 7.4, containing 1% Triton X-100 using a Teflon glass homogenizer. Subsequently, cell debris was removed by centrifugation for 5 min at 500 × g, 4°C. The activity of ASCT was determined by a radioactive assay as described previously (1). In brief, the assay mix contained 50 mM succinate, pH 7.4, 1 mM [1-14C]acetyl-CoA (0.2 Mbq, Amersham Biosciences), 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, and 0.05% (v/v) Triton X-100. The assay was started by the addition of the homogenate (50–100 μg protein), incubated at 20°C for 10 min, and terminated by the addition of ice-cold trichloroacetic acid (10% w/v, final concentration). Reaction products were then separated by anion-exchange chromatography and quantified by liquid scintillation counting.

Immunoprecipitation—Procyclic cells (1 × 10^6) were harvested by centrifugation at 500 × g for 5 min. The cells were washed twice and resuspended in 20 mM Tris-HCl buffer, pH 7.4, containing 1% Triton X-100. Antiserum was added to the cell lysate (20% w/v) and incubated on ice for 2 h. Subsequently, protein A-conjugated acryllic beads (Sigma, 150 μm) in 20 mM Tris-HCl buffer, pH 7.8, were added to the lysate and rotated for 2 h at 4°C. The antigen-bead preparation was pelleted by centrifugation (500 × g for 10 s). The supernatant was collected, and the pelleted beads were washed once with 20 mM Tris-HCl buffer, pH 7.8, and then resuspended in the same buffer. Both the supernatant and the resuspended beads were assayed for ASCT activity. Cell lysates treated with protein-A acryllic beads only were used as the control.

Nuclear Magnetic Resonance (NMR) Experiments—4 × 10^6 T. brucei procyclic cells grown in the SDM79 medium (up to 1 × 10^6 cells·ml−1) were incubated in 10 ml of incubation buffer (PBS buffer supplemented with 24 mM NaHCO3, pH 7.3) containing 110 μmol d-[1-13C]glucose (11 mM) for 90–180 min at 27°C as described before (11, 39). The cell viability and motility, checked every 30 min, were not affected during the incubation time. D-Glucose concentration in the medium was determined at the beginning and the end of the incubation with the D-glucose Trinder kit (Sigma). The supernatant was lyophilized and re-dissolved in 500 μl of D2O, and 15 μl of pure dioxane was added as an external reference. 13C NMR spectra were collected as described before (11, 39). The relaxation delay was 8 s for a nearly complete longitudinal relaxation. The spectrumspecific 13C enrichment of lactate (carbon C3), acetate (carbon C2), and succinate (carbons C2 and C3) was determined from 1H-observed/13C-edited NMR (1H/13C NMR) spectra acquired under 13C decoupling (40, 41) as described before (39). The reproducibility and accuracy of the method were assessed using pure solutions of succinate, acetate, lactate, malate, pyruvate, or β-hydroxybutyrate; the relative errors on the 13C enrichment determinations were <5%. The amount of 13C-enriched molecules (including n-glucose) was calculated on the basis of the dioxane peak. For each experiment, the amount of remaining d-glucose calculated from the NMR spectra corresponded ±5–25% to the concentration measured with the D-glucose Trinder kit.

Metabolic Incubations—Proliferating T. brucei cells (starting at 5 × 10^6 cells·ml−1) were incubated for 18–24 h at 27°C in sealed 25-ml Erlenmeyer flasks containing 5 ml of SDM79 incubation medium. Before sealing, the flasks were flushed for 1 min with a gas phase of air and 5% CO2 (95/5%). The incubations were performed after the addition of 5 μCi of [1-14C]glucose. Incubations were terminated by the addition of 40 μl of 6 M HCl to lower the pH to 3.5. Preceding acidification, 0.1 ml 1 M NaHCO3 was added through the rubber stopper, and the flasks were placed on ice. Immediately after acidification, the incubation flasks were flushed with nitrogen for 90 min at 0°C. In this way all carbon dioxide was removed, whereas acetate remained in the incubation medium. The carbon dioxide was trapped in a series of four scintillation vials, each filled with 1 ml of 0.3 M NaOH and 15 ml of Tritisol D2O, and 15 μl of pure dioxane was added as an external reference.

RESULTS

Identification of an ASCT Candidate Gene—The transfer of the coenzyme A (CoA) group is catalyzed by a large family of prokaryotic and eukaryotic CoA transferases (47) that share conserved protein domains (CoA trans in protein families data base, accession PF01144) and have a broad range of substrate specificities. Acetoacetate, a preferred substrate of mammalian succinyl-CoA3-ketoacid-CoA transferase, is structurally comparable with acetate, the substrate of ASCT. Therefore, we first searched for succinyl-CoA3-ketoacid-CoA transferase-related gene sequences in the available T. brucei genome databases using BLAST. Only a single high scoring segment pair was identified, and the target shares up to 55% amino acid identity over its full length with succinyl-CoA3-
ketoacid-CoA transferase from several vertebrate species. A single copy is located on chromosome 11 of the *T. brucei* genome reference strain TREU 9274 (TrypDB). The gene encodes a 53-kDa protein (493 amino acids) with two CoA transferase domains in tandem, the hallmark of eukaryotic CoA transferases (48). All nine amino acids found in immediate proximity of the catalytic center in the crystal structure of porcine succinyl-CoA:3-ketoacid-CoA transferase (48) are conserved in the new CoA transferase identified in *T. brucei*. Orthologues were also identified in *T. cruzi* (76% amino acid identity, GeneDB_Teruzi), and *Leishmania major* (65% amino acid identity, GeneDB_Lmajor).

**RNAi-mediated Repression of ASCT Activity**—To correlate expression of the ASCT candidate gene with ASCT activity, we generated procyclic *T. brucei* cell lines for inducible RNAi-mediated repression of the protein. A fragment of the candidate open reading frame was placed between inverted copies of a tetracycline-inducible T7 promoter and T7 polymerase for production of double-stranded RNA (2p3-1 cell line). At different time points after induction with tetracycline, the ASCT activity was measured as described before (Fig. 1B). Rabbit antibodies against the candidate open reading frame were produced after recombinant expression in *E. coli* (see “Experimental Procedures”) and were used for accurate quantitation of the protein by Western blotting followed by detection with infrared fluorescent probes (Odyssey system, Licor). The time course of RNAi-mediated repression shows an excellent correlation between the amount of the 53-kDa protein and ASCT activity (Fig. 1). After 96 h the level of the protein was at or below the limit of detection (3% of uninduced, determined from dilution series). The slow decline over four days indicated a relatively long half-life. Very similar data (Fig. 2) were also obtained with two independent clones (hp3 and hp4), resulting from a different strategy to produce double-stranded RNA, namely expression of an inverted repeat of the target sequence that folds into an RNA hairpin (construct pLew-ASCT-SAS). Immunoreactive protein and ASCT activity were both reduced 10-fold after 96 h of tetracycline induction. Note that the procyclic host lines and RNAi vectors are different in the two experiments shown in Figs. 1 and 2. Together, these independent data sets strongly suggest that the candidate gene encoded ASCT. An important function of ASCT was anticipated from the observed growth phenotype. The population doubling times of RNAi clones hp3 and hp4 increased from 10.7 to 15.4 h and from 11.1 to 15.3 h, respectively, upon tetracycline induction.

**Gene Deletion and Overexpression of T. brucei ASCT**—To confirm the identity of the ASCT gene, procyclic lines with targeted gene deletion of ASCT were generated. Replacement vectors with phleomycin and blasticidin resistance markers (BLE and BSD, respectively) were constructed and transfected, and correct homologous integration of the targeting vectors in the resulting drug resistant clones was analyzed by PCR using two independent primer pairs per locus type (wt, Δasct::BLE, Δasct::BSD). Whereas targeting of the first allele resulted in correct integration in 5 out of 5 clones, only 3 out of 52 independent double resistant clones were confirmed as homozygous gene deletions and were verified by Southern analysis (Fig. 3, A and B). This unusually low frequency of homologous integration and the low transfection efficiency in those experiments indicated strong selection against loss of the last ASCT gene copy. This interpretation was also supported by a severe growth phenotype of the three independent Δasct::BLE/Δasct::BSD knock out clones (Fig. 4). The initial population doubling time increased from 11 h (wt) to 18 h (clone 45), 27 h (clone 3.7), and 30 h (clone 3.23). Also, a higher fraction of aberrant cell divisions was revealed by microscopic scoring of 4′,6-diamidino-2-phenylindol-stained nucleus/kinetoplast configurations in those clones (not shown). The absence of ASCT protein on
Western blots (Fig. 3C) and a 4-5-fold reduced ASCT activity in knock out clones (Fig. 3D) confirmed deletion of ASCT. The significant residual enzyme activity measured by the ASCT assay in Δasct::BLE/Δasct::BSD knock out clones was due to an acetate-producing activity that is independent of succinate and, hence, distinct from ASCT (data not shown).

For genetic rescue of the Δasct/Δasct phenotype and stable overexpression of ASCT, we introduced into clone 45 (Δasct::BLE/Δasct::BSD) the construct pTSArib.ASCT, which integrates into the ribosomal promoter region. Quantitative Western analysis of dilution series documented 16-fold overexpression (Fig. 3C). This resulted in a 3-fold overexpression of ASCT activity (Fig. 3D), again confirming the correlation between ASCT expression and specific enzyme activity. Partially inative protein or posttranslational regulation of the activity may explain the disproportionality between ASCT protein level and enzyme activity upon overexpression. The rescued line had a normal population distribution of nucleus/kinetoplast configurations (not shown), but only partial reversion of the growth phenotype was achieved (Fig. 4). Because 8-fold overexpression in the wild type background resulted in a normal growth rate (not shown), the incomplete reversion of the growth phenotype of clone 45 is likely to be due to the selection history rather than to overexpression of ASCT.

Immunoprecipitation using the antiserum against the recombinant protein fragment (see “Experimental Procedures”) resulted in depletion of ASCT activity from cell lysates of wild type procyclic cells (98 ± 3% depletion) and of the transgenic clone overexpressing ASCT (79 ± 14% depletion). No activity could be detected in the resuspended acrylic beads fractions, which is probably due to binding of the polyclonal antibodies to the protein.

**Mitochondrial Localization of ASCT**—ASCT activity had been previously localized to mitochondria of procyclic T. brucei (1). Therefore, we determined the subcellular localization of the cloned ASCT gene product with two different methods, immunofluorescence microscopy and digitonin fractionation. Fig. 5 shows that anti-ASCT antibodies specifically stain a tubular structure, readily identified as the mitochondrion by the hsp60 marker in wt cells but not in Δasct/Δasct cells or in induced RNai knockdown cells. Wild type procyclic cells were also fractionated by increasing concentrations of digitonin, and ASCT protein was quantified by Western blotting in the soluble and insoluble fractions (Fig. 6). Established cytosolic (phosphoglyceraldehyde kinase) and mitochondrial (hsp60) markers were run as internal controls. The fractionation behavior of ASCT was virtually identical to that of hsp60, indicating a mitochondrial matrix localization. This was confirmed by digestion of selected digitonin fractions with two different proteases, proteinase K (not shown) and thermolysin (Fig. 6) before Western analysis. ASCT remained fully protected in the mitochondrial pellet fraction but was completely digested in the soluble fraction. In agreement with this localization, a short (11–12 amino acids) N-terminal mitochondrial target sequence was predicted by algorithms available via internet services (MitoProt II, TargetP, iPSORT) and by sequence alignment to vertebrate succinyl-CoA:3-ketoacid-CoA transferase precursors. The colocalization of ASCT activity (1) and the candidate gene product in the mitochondrion strengthens our evidence for identification and cloning of the bona fide ASCT gene.

**Analysis of Glucose Metabolism after Manipulation of the T. brucei ASCT Expression**—We used a carbon 13 (13C) NMR spectroscopy analysis to detect and quantify the metabolic end products excreted by the wild type and mutant cell lines. The parasites were incubated in PBS/NaHCO3 medium containing 11 mM D-[1-13C]-glucose as the only carbon source until up to half of D-glucose was consumed by each cell line. The incubation medium was then analyzed by NMR spectroscopy. The wild type AnTat1.1 and EATRO1125 procyclic cells mainly excrete 13C-enriched succinate, acetate, and lactate (~70, 18, and 7% of the 13C-enriched excreted molecules, respectively) with traces of malate, fumarate, and alanine (Fig. 7A and Table I) (11, 39). Surprisingly, the rate of acetate excretion in the AnTat1.1 and derived Δasct::BLE/Δasct::BSD (KO) cell lines was in the same range (122 and 132–136 nmol of 13C-enriched excreted molecules h−1 mg−1 protein, respectively), whereas the rate of succinate excretion increased by 17–66% in the KO cell lines (Table I). Consequently, the total amount of 13C-enriched end products excreted by the KO cell lines increased by 21–65%. To interpret these data the rate of glucose con-
Fig. 4. Long term growth of ASCT-deficient and rescued cell clones. Cells were cultured in SDM79 medium over 47 days and were regularly passaged by dilution to maintain the density between $5 \times 10^6$ and $1 \times 10^7$ cells ml$^{-1}$. The cumulative cell number is plotted in log scale on the y axis. The genotype of clones 45, 3.7, and 3.23 is Δasct::BLE/Δasct::BSD; the genotype of the clone 45-derived rescued line is Δasct::BLE/Δasct::BSD ASCT HYG. The culture time corresponding to the number of population doublings at the start of radioactive glucose incubations and NMR analysis is indicated.

Table I

| Excreted end products (numbers in Fig. 7) | Succinate (1) | Acetate (2) | Lactate (3) | Malate (4) | Fumarate (5) | Alanine (6) | Hydroxybutyrate (7) | Pyruvate (8) | TOTAL |
|-------------------------------------------|---------------|-------------|-------------|-----------|-------------|-------------|-------------------|-------------|-------|
| AnTat1.1 (n = 4)                          | 532 ± 31      | 122 ± 23    | 50 ± 21     | 27 ± 4.7  | 4 ± 1.2     | 7 ± 4.7     | ND                | ND          | 742 ± 76 |
| KO 45 (n = 5)                             | 620 ± 50      | 132 ± 27    | 55 ± 25     | 45 ± 8.0  | 9 ± 1.6     | 13 ± 5.5    | 19 ± 4.2          | ND          | 896 ± 125 |
| KO 3.7 (n = 2)                            | 857b          | 130b        | 40b         | 3.7b      | 10b         | 8b          | 25b               | 25b         | 1174b  |
| KO 3.23 (n = 2)                           | 884b          | 134b        | 49b         | 58b       | 11b         | 3b          | 25b               | 32b         | 1226b  |
| Rescue (n = 2)                            | 498b          | 188b        | 31b         | 52b       | 4b          | 3b          | ND                | ND          | 776b   |
| EATRO1125-T7T (n = 5)                     | 470 ± 88      | 142 ± 36    | 55 ± 15     | 20 ± 1.7  | 4 ± 1.1     | 8 ± 2.1     | ND                | ND          | 699 ± 160 |
| RNAi hp3 –tet (n = 3)                     | 655 ± 47      | 151 ± 10    | 76 ± 28     | 21 ± 5.0  | 4 ± 0.5     | 7 ± 1.4     | ND                | ND          | 914 ± 81 |
| RNAi hp3 + tet (n = 3)                    | 636 ± 116     | 129 ± 24    | 81 ± 34     | 31 ± 3.1  | 7 ± 0.4     | 11 ± 2.8    | 23 ± 6.1          | ND          | 918 ± 167 |
| RNAi hp4 –tet (n = 2)                     | 624b          | 157b        | 27b         | 24b       | 6b          | ND          | ND                | ND          | 896b   |
| RNAi hp4 + tet (n = 3)                    | 685 ± 57      | 146 ± 30    | 72 ± 6.3    | 53 ± 3.1  | 11 ± 1.2    | 12 ± 4.1    | 24 ± 2.3          | ND          | 1003 ± 115 |

| Excreted end products (numbers in Fig. 7) | Percentage of 13C-enriched excreted molecules | Succinate (1) | Acetate (2) | Lactate (3) | Malate (4) | Fumarate (5) | Alanine (6) | Hydroxybutyrate (7) | Pyruvate (8) | NMR\textsuperscript{c} | SDM79\textsuperscript{f} |
|-------------------------------------------|-----------------------------------------------|---------------|-------------|-------------|-----------|-------------|-------------|-------------------|-------------|-----------------|----------------|
| AnTat1.1 (n = 4)                          |                                               | 71.7          | 16.4        | 6.7         | 3.6       | 0.6         | 0.9         | ND                | ND          | 1.58 ± 0.12     | 1.36           |
| KO 45 (n = 5)                             |                                               | 69.3          | 14.7        | 6.5         | 5.0       | 1.0         | 1.4         | 2.1               | ND          | 2.19 ± 0.36     | 2.11           |
| KO 3.7 (n = 2)                            |                                               | 72.9          | 11.6        | 3.4         | 6.0       | 0.9         | 0.7         | 2.0               | ND          | 2.06b           | 2.00           |
| KO 3.23 (n = 2)                           |                                               | 72.2          | 10.9        | 4.0         | 7.2       | 0.9         | 0.2         | 2.0               | ND          | 2.06b           | 2.03           |
| Rescue (n = 2)                            |                                               | 64.2          | 24.3        | 4.1         | 6.7       | 0.5         | 0.2         | 2.1               | ND          | 1.77b           | 1.46           |
| EATRO1125-T7T (n = 5)                     |                                               | 67.3          | 20.3        | 7.9         | 2.8       | 0.6         | 1.1         | ND                | ND          | 1.68 ± 0.19     | 1.35           |
| RNAi hp3 –tet (n = 3)                     |                                               | 71.4          | 16.6        | 8.3         | 2.3       | 0.5         | 0.9         | ND                | ND          | 1.86 ± 0.20     | 1.70           |
| RNAi hp3 + tet (n = 3)                    |                                               | 69.3          | 14.0        | 8.8         | 3.4       | 0.8         | 1.2         | 2.5               | ND          | 1.96 ± 0.14     | 2.17           |
| RNAi hp4 –tet (n = 2)                     |                                               | 66.9          | 17.5        | 8.6         | 2.7       | 0.6         | 1.1         | ND                | ND          | 1.68b           | 1.43           |
| RNAi hp4 + tet (n = 3)                    |                                               | 68.5          | 14.6        | 7.1         | 5.3       | 1.1         | 1.2         | 2.3               | ND          | 1.92 ± 0.21     | 2.20           |

\textsuperscript{a} The mean of 2–5 experiments are presented; the results are shown ± S.D. when more than two experiments have been performed per cell line.
\textsuperscript{b} Duplicate, deviation of the mean is <20%.
\textsuperscript{c} Duplicate, deviation of the mean is between 20 and 40%.
\textsuperscript{d} Duplicate, deviation of the mean is >40%.
\textsuperscript{e} Rate of glucose consumption during the NMR experiments presented in this table.
\textsuperscript{f} Rate of glucose consumption by the same cell lines incubated for 8 h in SDM79 medium at $4 \times 10^6$ cells ml$^{-1}$.

The KO cell lines consumed significantly more glucose compared with the parental AnTat1.1 (Table I). This was measured during the incubation for NMR analysis (30–40% increase) as well as in separate incubations in SDM79 medium (47–55% increase). The ratio of acetate/succinate excretion was significantly reduced in the KO cell lines as compared with AnTat1.1 cells (10.9–14.7 versus 16.4% of the 13C-enriched excreted molecules, respectively). Thus, deletion of the ASCT gene resulted in a relative reduction of acetate production, although other activities must account for maintenance of acetate excretion in the absence of ASCT (see “Discussion”). The KO cell lines but not the wild type also excreted detectable amounts of 13C-enriched pyruvate and β-hydroxybutyrate (Fig. 7, A–B).

The rescued Δasct/Δasct cell line showed rates of glucose consumption and end product excretion similar to AnTat1.1, and no excretion of pyruvate and β-hydroxybutyrate was detected (Fig. 7C). As expected for overexpression of ASCT protein and activity (Fig. 3), the amount of excreted acetate significantly increased from 122 in the wild type to 188 nmol of
$^{13}$C-enriched excreted molecules $\cdot h^{-1} \cdot mg^{-1}$ protein in the rescued line (Table I). Clearly, the metabolic phenotype of $\Delta$asct/Δasct cells was fully rescued by extragenic ASCT expression, confirming the genetic link between the metabolic phenotype and ASCT activity.

Very similar metabolic changes were measured in tetracycline-induced RNAi hp3 and hp4 cells when compared with the parental EATRO1125-T7T cell line, i.e., an increase of the rates of glucose consumption and total end product excretion, a slight decrease of the ratio of acetate and succinate production, and excretion of some β-hydroxybutyrate (Table I). The non-induced RNAi hp3 and hp4 control cell displayed an intermediate metabolic phenotype. This was not surprising, as a certain leakiness of the tetracycline-inducible expression systems in $T. brucei$, which drives transcription of double-stranded RNA, is generally observed $^{26, 28, 39}$. Indeed, we quantified the amount of ASCT protein in all non-induced RNAi clones and found 50% of the protein level measured in the matched wild type cell lines (data not shown).

In addition to the $^{13}$C NMR studies, which could only be performed on harvested cells at high cell density in a buffered salt medium, we also investigated the end product formation in growing wild type and mutant procyclic cells under more physiological conditions. The major radioactive end products excreted during growth in SDM79 culture medium containing 6-$^{14}$C-labeled glucose were acetate and succinate as reported before $^{(12)}$. The total radioactivity of minor unidentified metabolites that were detected in these experiments never exceeded 10% of the sum of acetate and succinate. Therefore, only acetate and succinate are represented in Table II. The absence of ASCT did not result in a major change in the ratio of acetate and succinate produced, although a slight but not significant decrease in acetate production is suggested by the comparison of the wild type and $\Delta$asct/Δasct KO 45 cells (Table II). This is in agreement with the $^{14}$C-glucose incubations performed with RNAi clone 2p3.1 (Fig. 1) and also with the $^{13}$C NMR analysis of end products excreted by $\Delta$asct/Δasct clones and RNAi clones hp3 and hp4 (Table I). The estimated $^{14}$C-glucose degradation (calculated from the sum of excreted products) was not increased in KO 45 cells compared with the wild type parent (Table II). It should be noted that the experimental conditions of $^{14}$C-glucose incubations and $^{13}$C NMR analysis are very different and that KO 45 showed the weakest metabolic phenotype of several $\Delta$asct/Δasct clones (see Table I).

Analysis of the KO 45 rescue line that overexpresses ASCT activity (see Fig. 3) showed a significant increase in acetate production, whereas succinate production was decreased (Table II). This is again in agreement with the $^{13}$C NMR studies (Table I) and provides direct evidence for an important role of ASCT in acetate production under physiological conditions.

**DISCUSSION**

ASCT enzyme activity in $T. brucei$ is encoded by a newly identified member of the eukaryotic CoA transferase gene family. This is the first report of a gene encoding ASCT. Several lines of evidence support the gene-to-activity assignment. 1) The completed genome sequence of $T. brucei$ contains only one member of the highly conserved and well recognizable CoA transferase gene family I $^{(47)}$. 2) A quantitative correlation was documented between ASCT activity and the amount of protein encoded by the candidate ASCT gene. Inducible RNAi-mediated repression with two independent methods was used. 3) Homozygous-targeted deletion of the ASCT gene resulted in protein product and activity levels below the experimental background. 4) Overexpression of the candidate gene product in $T. brucei$ resulted in increased ASCT activity and increased acetate production. 5) Both the candidate gene product and ASCT activity are expressed in procyclic form $T. brucei$ but not in the bloodstream stage of the parasite (data not shown). 6) Mitochondrial localization of the candidate ASCT gene product was verified by two independent methods and corresponds to mitochondrial localization (1) of the ASCT activity. 7) ASCT

![Fig. 5. Subcellular localization of ASCT by immunofluorescence analysis](image)

![Fig. 6. Localization of ASCT by subcellular fractionation](image)
activity could be removed by immunoprecipitation with a specific antiserum raised against the candidate ASCT gene product. Unfortunately, heterologous expression of ASCT protein in E. coli, Saccharomyces cerevisiae, and Pichia pastoris resulted in insoluble protein with no detectable activity. In procyclic T. brucei, acetyl-CoA produced from glucose is converted into acetate and is not metabolized through the Krebs cycle (12). Van Hellemond et al. (1) previously characterized a mitochondrial ASCT activity in trypanosomatids involved in acetate production from acetyl-CoA. Here, we have investigated the role of ASCT for acetate production in wild type and genetically engineered trypanosomes by qualitative and quantitative analyses of the excreted end products of glucose metabolism. To our surprise the level of acetate excretion was only slightly reduced in the mutant cell lines devoid of ASCT activity, suggesting an additional pathway(s) involved in acetate production from glucose. In fact, a minor succinate-independent acetate production from acetyl-CoA was observed before (1). This activity could be increased in the Δasct/Δasct cell lines as an adaptive change taking place during selection of the knock out lines. Although acetate excretion is only slightly reduced, the mutant cell lines show a distinct metabolic phenotype of ASCT deficiency. 1) The Δasct/Δasct cell lines excrete pyruvate and β-hydroxybutyrate, which are not detectable in the incubation medium of the wild type. In mammals, β-hydroxybutyrate is the end product of a four step pathway used to eliminate excess of acetyl-CoA. Because possible orthologues of the respective enzymes are present in the T. brucei genome sequence (data not shown), a β-hydroxybutyrate-producing pathway is likely to be present in this parasite. In addition, pyruvate is the precursor of the ASCT substrate acetyl-CoA. Thus, the excretion of both β-hydroxybutyrate and pyruvate, ...

**FIG. 7.** Carbon 13 NMR spectra of metabolic end products excreted by procyclic cell lines incubated with D-[1-13C]glucose. To perform this NMR analyses, the wild type AnTat1.1 (panel A), Δasct/Δasct clone 3.7 (panel B), and the overexpressing rescued clone 45 [pT-SARib.ASCT] (panel C) were incubated with 11 mM D-[1-13C]glucose in PBS/NaHCO₃ buffer. The NMR spectra were obtained after the addition of 15 μl of dioxane. Each spectrum illustrates one representative experiment from a set of 2–4. To visualize the pyruvate and β-hydroxybutyrate peaks, an extended view of the area between 20 and 32 ppm is presented in the inset. The β-hydroxybutyrate peak was verified by the addition of the compound as internal control (not shown). The resonances were assigned as follows: 1, succinate; 2, acetate; 3, lactate; 4, malate; 5, fumarate; 6, L-alanine; 7, β-hydroxybutyrate; 8, pyruvate; G, D-glucose (α and β); D, dioxane.
may be the consequence of acetyl-CoA accumulation in those mutant cells, which are less efficient in converting acetyl-CoA into acetate due to the absence of ASCT. Our interpretation is that the alternative acetate producing pathway(s) may not suffice to metabolize all the produced acetyl-CoA. 2) The rate of glucose consumption in the absence of ASCT may not be slow enough to metabolize all the produced acetyl-CoA. 3) The RNAi and KO mutant cell lines show significant reduction of their growth rate, which demonstrates that ASCT activity is important for normal functioning of procyclic T. brucei. The metabolic phenotype was fully rescued, and the growth phenotype was partially rescued by exogenous ASCT expression.

Together, the phenotype of ASCT deficiency, which seems to be due to accumulation of acetyl-CoA, and the fact that overexpression of ASCT resulted in a major increase of acetate excretion in proliferating parasites, leave no doubt that ASCT is a major source for acetate production in the normal situation. Although ASCT is not an essential gene and an alternative acetate-producing pathway(s) exists, the importance of the ASCT for physiological functioning is clearly shown. 1) The recovery of drug resistant clones upon targeting of the second allele was very slow, and 2) we observed that in most of these drug-resistant clones the targeting vector was not correctly integrated in the ASCT locus, a very rare event in T. brucei, where homologous recombination is the rule. Although Δasct Δasct cells are viable, short term adaptation does not overcome the phenotype discussed above. Upon continuous passage for an extended period, Δasct Δasct trypanosomes are finally able to recover the wild type growth rate (Fig. 4). The basis of this long term adaptation is not known.

In conclusion, our genetic approaches, the localization studies, and the two independent metabolic studies all show that the identified gene codes for the ASCT enzyme in T. brucei. Acetate production via ASCT occurs in many parasites (protozoa as well as helminths) but is absent in their vertebrate hosts. The enzyme seems, therefore, to be a logical target for broad-spectrum anti-parasitic drugs. This strategy will not be useful to combat African trypanosomiasis, because ASCT is not detected in the bloodstream stage of T. brucei infecting the mammalian host. ASCT is, however, present in infective stages of other trypanosomatids including Leishmania. The gene sequence of T. brucei ASCT will facilitate identification of ASCT in anaerobic protists such as N. ovalis, Neocallimastix, and T. vaginalis, which contain the various types of hydrogenosomes. Phylogenetic studies on ASCT, one of the few enzymes shared by hydrogenosomes and mitochondria of some species, could shed more light on the evolutionary relationships between mitochondria and the various types of hydrogenosomes.

Acknowledgments—We thank Najib M. A. El-Sayed, Rockville, MD for providing genome sequence information to the community and for plasmid 7D10 from the GSS library generated at The Institute for Genomic Research, Paul Michels, Brussels, for the generous supply of anti-phosphoglycerate kinase C serum, and Luc Vanhamme and Etienne Pays, Brussels, for vector pTSArib, Bianca Bauer and Alexandra Neukam (M. Boshart laboratory) contributed by construction of p2T7.ASCT and bioinformatic analysis, respectively. Jacques Bedaux, Utrecht, is acknowledged for his mathematical analysis of 14C product formation.

REFERENCES

1. Van Hellemont, J. J., Opprwoorl, F. R., and Tielens, A. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3036–3041
2. Opprwoorl, F. R. (1994) J. Bioenerg. Biomembr. 26, 145–146
3. Hanneveld, V., Brinkaud, F., Opprwoorl, F. R., and Michelez, P. A. (2003) Kinetoplastid Biol. Dis. 2, 11
4. Cazzulo, J. J. (1992) FASEB J. 6, 3153–3161
5. Blum, J. J. (1994) Parasitol. Today 9, 118–122
6. Opprwoorl, F. R. (1995) Biochemistry and Molecular Biology of Parasites (Marr, J. M., and London, A., eds) pp. 19–31, Academic Press, Inc., London, UK
7. Van Hellemont, J. J., Van Der Meer, P., and Tielens, A. G. M. (1997) Parasitology 114, 351–360
8. Fairbairn, A. H., and Opprwoorl, F. R. (1986) in Carbohydrate Metabolism in Cultured Cells (Morgan, J. M., ed) pp. 183–224, Plenum Publishing Corp., New York
9. Opprwoorl, F. R. (1987) Annu. Rev. Microbiol. 41, 127–151
10. Clayton, C., and Michels, P. A. (1996) Parasitol. Today 12, 465–471
11. Beste, S., Biron, M., Bizeau, N., Costant, V., Balz, T., Canioni, P., and Brinkaud, F. (2002) J. Biol. Chem. 277, 38001–38012
12. Van Weelden, S. W., Fast, B., Vogt, A., Van Der Meer, P., Saas, J., Van Hellemont, J. J., Tielens, A. G., and Boshart, M. (2003) J. Biol. Chem. 278, 12854–12863
13. Saz, H. J., deBruyn, B., and de Mata, Z. (1996) J. Parasitol. 82, 694–696
14. McLaughlin, G. L., Saz, H. J., and deBruyn, B. S. (1986) Comp. Biochem. Physiol. B 83, 525–527
15. Steinbuechel, A., and Mullen, M. (1986) Mol. Biochm. Parassitol. 20, 57–65
16. Marvin-Sikkema, F. D., Pedro Gomes, T. M., Grivet, J. P., Gottschalk, J. C., and Prins, R. A. (1993) Arch. Microbiol. 160, 388–396
17. van Hoek, A. H., Akhmanova, A. S., Huynen, M. A., and Hackstein, J. H. (2000) Mol. Biol. Evol. 17, 202–206
18. Emblle, T. M., Finlay, B. J., Dal, P. L., Hirt, R. P., Wilkinson, M., and Williams, A. G. (1995) Proc. Res. Soc. Lond. B 262, 97–93
19. Carrier-Smith, T., and Chao, E. E. (1996) J. Mol. Evol. 43, 551–562
20. Bui, E. T., Bradley, P. J., and Johnson, P. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9651–9656
21. Gernot, A., Philipp, H., and Le Guyader, H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14614–14617
22. Horner, D. S., Hirt, R. P., Klevling, S., Lloyd, D., and Emblle, T. M. (1996) Proc. Res. Soc. Lond. B 263, 1053–1058
23. Roger, A. J., Clark, C. G., and Doultile, W. F. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14618–14622
24. Martin, W., and Muller, M. (1996) Nature 392, 37–41
25. Kimura, M., Takatsuki, A., and Yamaguchi, I. (1994) Biochim. Biophys. Acta 1219, 653–659
26. Wirtz, E., Leal, S., Ochatt, C., and Cross, G. A. (1999) Mol. Biochem. Parasitol. 99, 89–101
27. LaCount, D. J., Bruse, S., Hill, K. L., and Donelson, J. E. (2000) Mol. Biochem. Parasitol. 111, 67–76
28. Bringaud, F., Robinson, D. R., Barradeau, S., Biteau, N., Baltz, D., and Baltz, T. (2000) Mol. Biochem. Parasitol. 111, 283–297
29. Xong, H. V., Vanhamme, L., Chamekh, M., Chimfwembe, C. E., Van Den Abbeele, J., Pays, A., Van Meervenne, N., Hamers, R., De Baetselier, P., and Pays, E. (1998) Cell 95, 839–846
30. Brun, R., and Schonenberger, M. (1979) Acta Trop. 36, 289–292
31. Delauw, M. F., Pays, E., Steinert, M., Aerts, D., Van Meervenne, N., and Le Ray, D. (1985) EMBO J. 4, 989–993
32. Vassella, E., and Boshart, M. (1996) Mol. Biochem. Parasitol. 82, 91–105
33. McCulloch, R., Vassella, E., Burton, P., Boshart, M., and Barry, J. D. (2004) Methods Mol. Biol. 262, 53–86
34. Harlow, E., and Lane, D. (eds) (1988) Antibodies: A Laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor Harbor, NY
35. Bringaud, F., Peyruchaud, S., Baltz, D., Biran, M., Diolez, P., Bouchaud, V., Voisin, P., Michels, P. A., Canioni, P., Baltz, T., and Bringaud, F. (2003) J. Biol. Chem. 278, 49625–49635
36. Bringaud, F., Peyruchaud, S., Baltz, D., Giroud, C., Simpson, L., and Baltz, T. (1995) Mol. Biochem. Parasitol. 74, 119–123
37. Saas, J., Ziegelbauer, K., von Haeseler, A., Fast, B., and Boshart, M. (2000) J. Biol. Chem. 275, 2745–2755
38. Sveshnikova, N., Grimm, R., Soll, J., and Schleiff, E. (2000) Biol. Chem. 381, 687–693
39. Coustou, V., Besteiro, S., Biran, M., Diolez, P., Bouchaud, V., Voisin, P., Michels, P. A., Canioni, P., Baltz, T., and Bringaud, F. (2003) J. Biol. Chem. 278, 49625–49635
40. Freeman, R., Mareci, T. H., and Morris, G. A. (1981) J. Magn. Reson. 42, 341–345
41. Rothman, D. L., Behar, K. L., Hetherington, H. P., den Hollander, J. A., Bendall, M. R., Petroff, O. A., and Shulman, R. G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1633–1637
42. Pande, S. V. (1976) Anal. Biochem. 74, 25–34
43. Horemans, A. M., Tielens, A. G., and van den Bergh, S. G. (1991) Parasitology 102, 259–265
44. Tielens, A. G., van der Meer, P., and van den Bergh, S. G. (1981) Mol. Biochem. Parasitol. 3, 205–214
45. Bergmeyer, H. U., Bernt, E., Schmidt, F., and Stork, H. (1970) in Methoden der Enzymatischen Analyse (Bergmeyer, H. U., ed) pp. 1163–1165, Verlag Chemie, Weinheim, Germany
46. Bensadoun, A., and Weinstein, D. (1976) Anal. Biochem. 70, 241–250
47. Heider, J. (2001) FEBS Lett. 509, 345–349
48. Bateman, K. S., Brownie, E. R., Wolofsky, W. T., and Fraser, M. E. (2002) Biochemistry 41, 14455–14462

Acetyl:Succinate CoA-transferase
