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Knockout of the glutamate dehydrogenase gene in bloodstream *Trypanosoma brucei* in culture has no effect on editing of mitochondrial mRNAs☆

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Abstract

Glutamate dehydrogenase (GDH) was shown previously to bind the 3′ oligo[U] tail of the mitochondrial guide RNAs (gRNAs) of *Leishmania tarentolae*, apparently in the dinucleotide pocket (Bringaud F, Stripecke R, Frech GC, Freedland S, Turck C, Byrne EM, Simpson L. Mol. Cell. Biol. 1997;17:3915–3923). Bloodstream *Trypanosoma brucei* cells in culture represent a good system to investigate the genetic effects of knocking out kinetoplastid nuclear genes to test a role in RNA editing, since editing of several mitochondrial genes occurs but is dispensable for viability (Corell RA, Myler P, Stuart K. Mol. Biochem. Parasitol. 1994;64:65–74 and Stuart K. In: Benne R, editor. RNA editing—the alteration of protein coding sequences of RNA. New York: Ellis Horwood, 1993:25–52). Both GDH alleles of bloodstream *T. brucei* in culture were replaced by drug resistant markers without any effect on viability. The ratios of edited to unedited mRNAs for several cryptogenes were assayed by primer extension analysis. The steady state abundances of these edited RNAs were unaffected by the double knockout. This evidence suggests that GDH may not play a role in the editing reaction in bloodstream trypanosomes in culture, but this conclusion is tentative since there could be redundant genes for any biological function. We employed a double allelic replacement technique to generate a tetracycline inducible conditional expression of an ectopic copy of the deleted gene in bloodstream trypanosomes in culture. We used this strategy for genes encoding mitochondrial proteins which are not required

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during this stage of the life cycle, but as a general strategy it should be appropriate for generation of conditional null mutants for essential genes as well.

Keywords: Kinetoplast DNA; *Trypanosoma brucei*; Glutamate dehydrogenase; Knockouts; RNA editing

1. Introduction

The mitochondrial metabolic enzyme glutamate dehydrogenase (GDH), was shown previously to represent a major guide RNA(gRNA)-binding protein in *Leishmania tarentolae* [1]. A major determinant of the gRNA recognition is the 3′ oligo[U] tail. The interaction apparently involves the NADP(H)-binding pocket of the enzyme since the binding could be specifically competed with this dinucleotide. The mitochondrial GDH also was shown to bind UTP and this binding was competed by NADP(H). The fact that in vitro uridine insertion RNA editing activities detected in mitochondrial extracts from *L. tarentolae* could also be inhibited by high concentrations of NADP(H) raised the possibility that GDH might be involved in the editing reaction.

Several metabolic enzymes that utilize dinucleotides as cofactors were found previously to bind RNA within the dinucleotide pocket, and it was proposed that these enzymes represent a novel class of RNA-binding proteins with a diverse range of biological functions [4,5]. Hentze has speculated [4] that metabolic RNA-binding enzymes may participate in general regulatory circuits linking metabolic and genetic functions. In view of the reported regulation of RNA editing during the life cycle of trypanosomes [3,6], it was attractive to speculate that the binding of gRNA and/or UTP by GDH represented a possible link between cell metabolism and editing [1].

In this paper we present gene knockout evidence with *Trypanosoma brucei* bloodstream trypomastigote cells in axenic culture possibly arguing against a role for GDH in RNA editing, at least in this stage of the life cycle.

2. Materials and methods

2.1. Cell culture

T. brucei strain 427 MiTat 1.2 cells were grown as bloodstream forms in HMI-9 medium [7] at 37°C in 5% CO_2. The method of Hesse et al. [8] was used to increase the yield of cells in culture at 37°C. Cells were centrifuged daily and the medium replaced, for a total of 53 days. This resulted in stationary phase cultures ($5–10 \times 10^6$ cells/ml) which did not die rapidly as occurred with the wild type cells. However, this cell line could no longer differentiate into procyclic cells even with the addition of *cis*-aconitate and epithelial feeder cells [9].

2.2. DNA analysis

The degenerate primers used for PCR amplification of a fragment of the *T. brucei* GDH gene are as follows: 5′-AA(GA) AA(CT) AA(GA) GA(TC) AA(ATC) CC(GATC) GA(AG) GG(GATC) and 5′-(GATC)GT (GATC)AC (GATC)CC (GATC)CC (TC)TT (AG)TT (GATC)GC (degenerate bases are in parentheses). This fragment was used for hybrid selection of a *T. brucei* genomic cosmid clone. The GDH open reading frame and untranslated regions were sequenced using custom oligodeoxynucleotides and the Sequenase DNA Sequencing Kit (United States Biochemicals, Amersham) or the ABI PRISM Dye Terminator Cycle Sequencing kit.

The genomic DNA used for Southern analysis and PCR amplification was isolated from parasites using the method described for mammalian cells [10]. For Southern analysis, the DNA was

loaded in a 0.6%-TBE agarose gel, electrophoresed, transferred to Zeta-Probe membranes (BioRad) and hybridized to radiolabeled DNA probes according to the manufacturer's instructions.

2.3. Construction of targeting vectors and transfection of trypanosomes

The GDH gene flanking regions (UTRs) used to target both GDH alleles were PCR-amplified using genomic DNA as template. The 5′ UTR (nucleotides 365–634) was obtained using the 5′ oligodeoxynucleotide S2196 (5′ GGGCGGCCGCTTAGATTCTGTCACCTATTTTGC 3′, *Not*I site underlined) and the 3′ oligodeoxynucleotide S2197 (5′ GGCTCGAGACCGGTAATGCTTCGATATTGAATC, *Xho*I and *Age*I sites underlined). Two 3′ UTRs with different lengths were used. A short 3′ UTR (including nucleotides 4058–4580) was amplified using the 5′ oligodeoxynucleotide S2198 (5′ GGAGGCCTTGCTTGCGTTTGTAACCAACTG 3′, *Stu*I site underlined) and the 3′ oligodeoxynucleotide S2199 (5′ GGGCTAGCGCGGCCGCTTCTCCCCCTTTCCCACTACTT 3′, *Nhe*I and *Not*I sites underlined). A longer version (including nucleotides 3828–4580) was also obtained by PCR amplification with the 5′ oligodeoxynucleotide S2365 (5′ GGAGGCCTGAAAAACGAGTGAAAGGGCATTAGC 3′, *Stu* I site underlined) and the 3′ oligodeoxynucleotide S2199.

The plasmid pKO1b (Fig. 1) is a pHD360 derivative [11] in which the *Not*I–*Xho*I fragment of β-tubulin was replaced with the GDH 5′ UTR, and the short GDH 3′ UTR was inserted between the *Stu*I and *Nhe*I sites.

To make pKO2b (Fig. 1), the neomycin resistance gene (NEO) cassette from pHD103 [12] was cloned as a *Acc*I–*Stu*I fragment in pKO1b, and the short GDH 3′ UTR was replaced with the long 3′ UTR.

The plasmid pLew47 (Fig. 1) is a derivative of pLew20 [11] in which the luciferase gene was replaced with a chloramphenicol acetyl transferase (CAT) gene (Wirtz et al., submitted for publication).

The pT7KO1c vector was constructed by replacing the tetracyline repressor (TetR) cassette from pKO1b with a *Bst* 1107I–*Bam*HI cassette containing a T7 RNA polymerase cassette from pHD328 [13]. The pGEM-derived T7 promoter present in pKO1b was previously removed by *Pvu*II digestion followed by religation.

pT7CAT1 (Fig. 1) is a pLew82 derivative [14] without T7 terminators, in which the luciferase gene was replaced with the CAT gene.

To make pKO2e (Fig. 1), we first replaced the *Bpu*AI–*Stu*I cassette containing the hygromycin resistance gene (HYG) from pLew90 (Wirtz et al., submitted for publication) with a NEO cassette flanked by actin processing signals from pHD102 [12], yielding the intermediate construct pLew90/Neo (Fig. 1). The TetR/NEO cassette from pLew90/Neo was then excised as a *Xho*I/*Stu*I fragment and cloned in pKO2b, yielding pKO2d. The pGEM-derived T7 promoter present in the cloning vector pKO2b was previously removed by *Pvu*II digestion followed by religation. Finally, the NEO cassette containing actin processing signals in pKO2d was replaced with a NEO cassette with aldolase processing signals by replacing the *Sma*I–*Nhe*I fragment with the *Xho*I (filled-in)-*Nhe*I fragment from pKO2b.

To transfect *T. brucei* trypomastigotes in culture at 37°C, plasmids were digested with *Not*I and the DNA fragments containing the targeting cassettes were isolated from agarose gels using the GeneClean kit (Bio101). Cells were electroporated and selected as described [14]. The double knockout cell lines were cloned in agarose plates [15]. CAT assays were performed in 2×10^6 cells using the FAST CAT (deoxy) chloramphenicol acetyltransferase assay kit (Molecular Probes), as recommended by the manufacturer. Spots were quantified using a UVP ImageStore apparatus and the ImageQuant software.

2.4. Northern and immunoblot analyses

Total cell RNA was isolated following the guanidium/phenol protocol [16]. Poly-A^+ selection was carried out using oligo(dT) cellulose chromatography according to Sambrook et al. [17]. For Northern analysis, 25 μg of total RNA

were electrophoresed in 1% agarose-formaldehyde gels, transferred to Zeta-Probe membranes and hybridized to radiolabeled probes following the manufacturer's instructions.

For Western blotting analysis, 7×10^6 cells were washed in phosphate-buffered saline and resuspended in 40 μl of Laemmli buffer. After heating 10 min at 95°C, the samples were briefly sonicated and loaded in a 8% polyacrylamide-SDS gel. Protein bands were transferred to nitrocellulose filters (Protran, Schleicher and Schuell) and immunodetected using the SuperSignal CL-HRP substrate system (Pierce) as instructed by the manufacturer.

2.5. Determination of the 5′ splice sites of GDH transcripts

The 5′ splice sites were mapped by 3′-nested RT-PCR [18]. cDNA was synthesized using poly-A^+ RNA and the oligodeoxynucleotide S2386 (5′ GGTTTCCCTTTCGCGGCTGACAAACTTGG

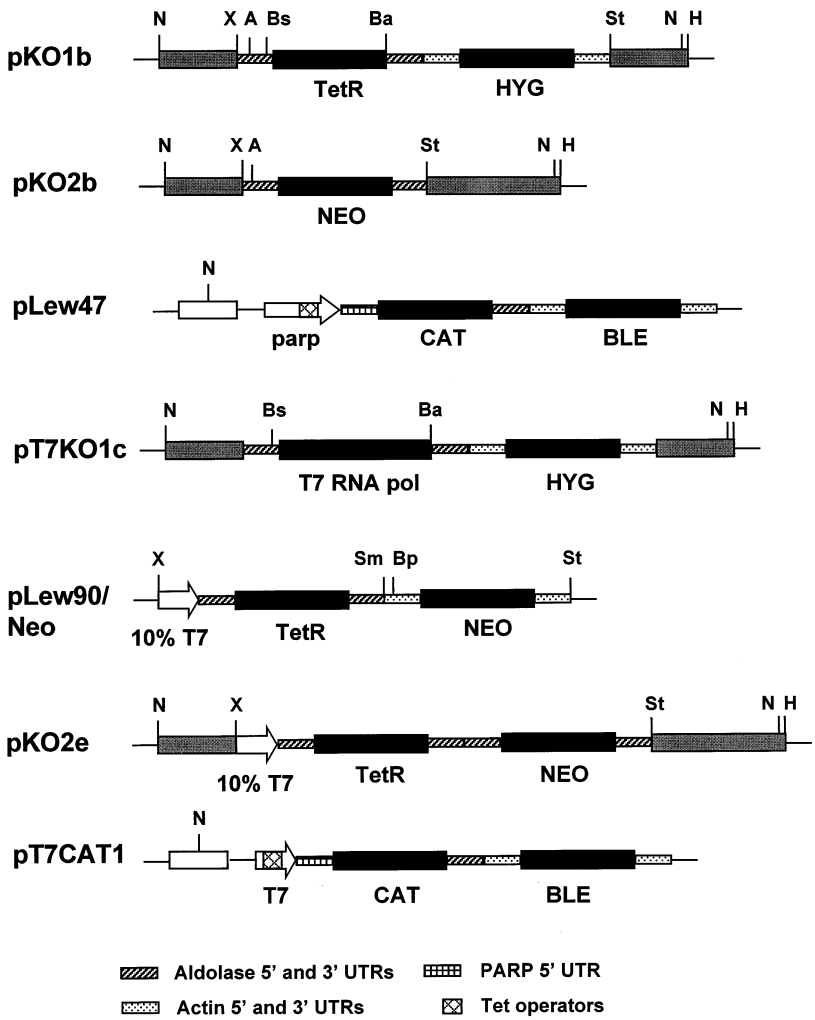

Fig. 1. Plasmid constructs used in this paper. Large black-filled boxes are open-reading frames, grey-filled boxes indicate GDH 5′ and 3′ UTRs, small filled boxes are processing signals and open boxes represent the rDNA intergenic spacer. Arrows indicate the transcriptional orientation of PARP and T7 promoters. N, *Not*I; X, *Xho*I; A, *Acc*I; Bs, *Bst*1107I; Ba, *Bam*HI; St, *Stu*I; H, *Nhe*I; Sm, *Sma*I; Bp, *Bpu*AI.

3′), complementary to nucleotides 1034–1062 of the GDH gene sequence. The cDNA was used as a template in a first round of PCR with the 5′ oligodeoxynucleotide S2385 (5′ GACGGATCCGCTATTATTAGAACAGTTTCTGTACTATATTG 3′, *Bam*HI site underlined), complementary to the residues 8–39 of the *T. brucei* miniexon and the 3′ oligodeoxynucleotide S2386. A second round of PCR amplification was carried out using 0.01 vol of the first PCR reaction as template, the 5′ oligodeoxynucleotide S2385 and the 3′-nested oligodeoxynucleotide S2387 (5′ CTCGCAGCTGACTTGCCTGGTCCTTTCG 3′), complementary to nucleotides 991–1018 of the GDH gene. The PCR products were analyzed in a 2% agarose-TBE gel, purified, cloned and sequenced to determine the miniexon splice site junctions.

2.6. Primer extension analysis

Total RNA (50 μg) was co-precipitated with 300 000 cpm of radiolabeled oligodeoxynucleotides, resuspended in 15 μl of hybridization buffer (10 mM Tris–HCl, pH 8.3, 150 mM KCl) and incubated for 90 min at a temperature (T_{hyb}) calculated from the formula $T_{hyb} = 64°C + 0.4(\%G + C) - 800/L$, where L is the length of the oligodeoxynucleotide. The cDNA synthesis was started by adding 15 μl of a mix containing 90 mM Tris–HCl (pH 8.3), 6 mM $MgCl_2$, 2 mM DTT, 0.5 mM each deoxynucleotide, 3 U of RNase inhibitor (Pharmacia) and 200 U of Superscript II reverse-transcriptase (BRL). After incubation for 1 h at 50°C, the reactions were stopped by adding 25 mM EDTA. The RNA was hydrolyzed by heating the samples 30 min at 65°C in the presence of 0.2 M NaOH. After adding an equivalent amount of HCl, the extension products were precipitated with 2 M ammonium acetate, 20 μg of glycogen as carrier and three volumes of ethanol. The pellets were washed with 70% ethanol, resuspended in loading solution (50% formamide, 0.03% bromophenol blue and xylene cyanol FF), heated 5 min at 95°C and loaded in a 6% acrylamide/urea sequencing gel. The extension products were quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics).

3. Results

3.1. Sequencing of the T. brucei GDH gene

A cosmid clone containing the *T. brucei* GDH gene was selected from a genomic library from the AnTat1.1 strain by hybridization using a fragment of the GDH gene which was PCR amplified from total cell DNA using degenerate primers generated by back translation of two conserved amino acid sequences from the *L. tarentolae* GDH (see Fig. 2, bold faced sequences). A 2.8 kb *Sma*I/*Hind*III fragment was selected by Southern blot hybridization, cloned and sequenced. This contained a portion of the GDH gene and the remainder was sequenced directly from the cosmid DNA by primer walking. Both 5′ and 3′ UTR sequences (666 nt 5′ and 965 nt 3′) were obtained for construction of the knockout vectors. Two splice acceptor sites were identified by RT-PCR in the 5′ flanking UTR for trans-splicing of the spliced leader sequence at positions 426 and 509.

An alignment of the *T. brucei* and the *L. tarentolae* GDH amino acid sequences is shown in Fig. 2. The sequences showed an overall percent identity of 53.9% and a percent similarity of 61.1%. There was little conservation at the amino terminal ends, as is the case with other GDH sequences, but there was considerable conservation around functionally important sites such as the NAD-binding site and there is no doubt that the *T. brucei* sequence represents a GDH homologue. The dinucleotide-binding site has been proposed to be involved in the binding of gRNA in *L. tarentolae* [1] and its conservation in *T. brucei* strongly suggests an identical role in the latter organism. Southern analysis showed that this is a single copy gene as in *L. tarentolae* (data not shown).

3.2. Strategy I: replacement of the first GDH allele with the TetR and HYG genes and replacement of the second allele with NEO

The initial strategy was to replace GDH allele 1 with the TetR gene together with HYG as a selectable marker, using the endogenous transcriptional activity of the GDH locus for expres-

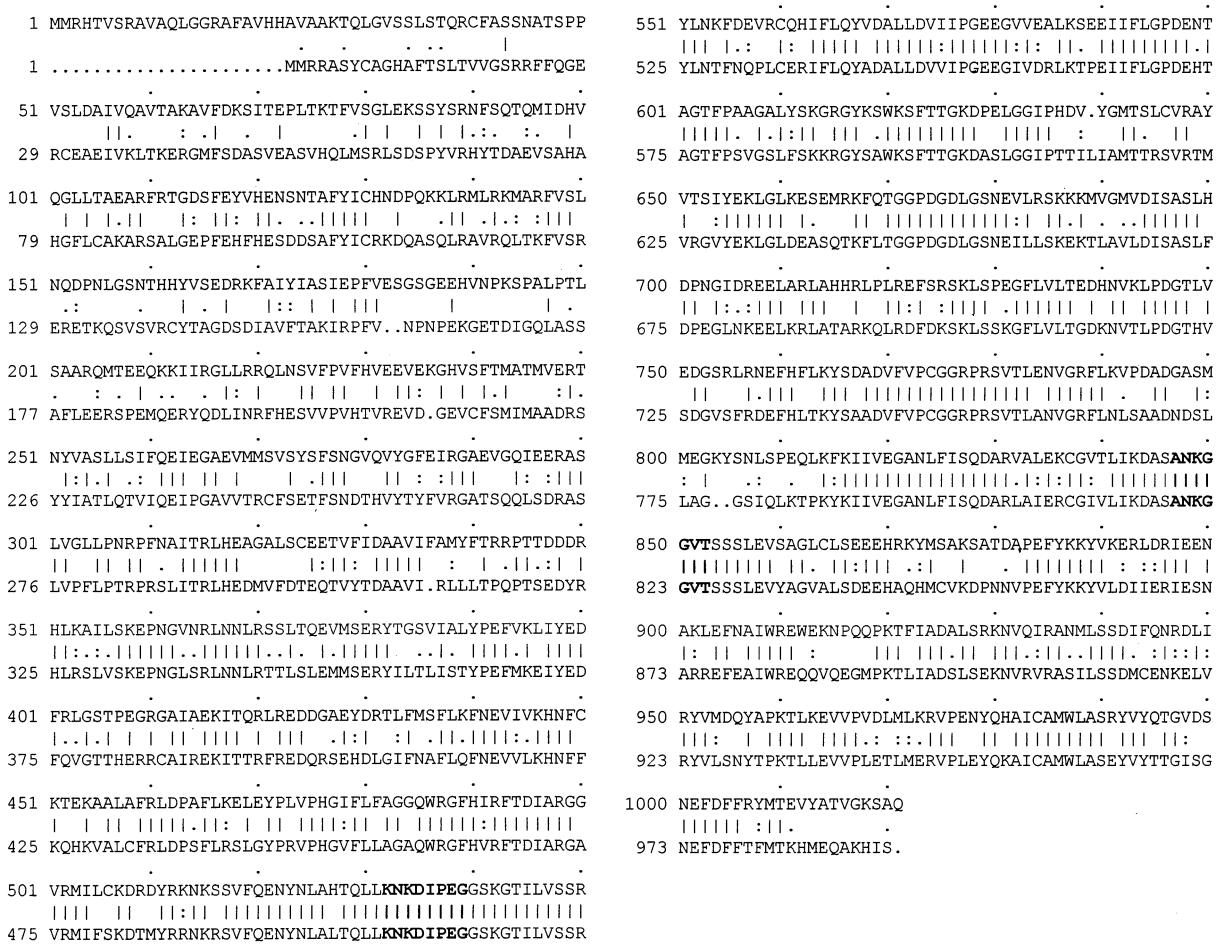

Fig. 2. Alignment of the *L. tarentolae* and *T. brucei* GDH amino acid sequences. A global alignment was performed using the GCG GAP program. The upper sequence is the *L. tarentolae* GDH and the lower sequence the *T. brucei* GDH. The conserved sequences used for primer construction are in bold face.

sion of the TetR. The second allele would then be replaced by NEO, and then an ectopic copy of the GDH gene controlled by a PARP promoter [19,20] containing tetracycline (Tet) operators [11] would be inserted into a silent locus using the phleomycin resistance gene (BLE) as a selectable marker. This should then allow the regulation of expression of the ectopic copy of the GDH with Tet.

The construction of the vectors for the first knockout is described in Section 2 and Fig. 1. Upstream and downstream flanking sequences of the GDH gene were used to allow gene replacement of GDH allele 1 with both the TetR and the HYG genes. Wild-type cells were electroporated with the pKO1b cassette (Fig. 1) and selected in the presence of 1 μg/ml of hygromycin. The integration of the TetR/HYG cassette was confirmed by Southern blot analysis (data not shown).

Two functional assays were performed to test expression of the TetR in this locus (Fig. 3). In the first assay, the KO-1 cells were transfected with supercoiled pLew47 (Fig. 1) that contains the CAT reporter gene under control of the PARP promoter with tet operators. As shown in Fig. 3A, two independent KO-1 clonal lines of *T. brucei*, named KO1(1) and KO1(2), exhibited a repression of transient CAT activity in the absence of

Tet and a derepression in the presence of this compound. There was however approximately a 5% background level of CAT expression in the absence of Tet, indicating a leaky control of expression.

To perform the second assay, we targeted the CAT gene under the control of the PARP promoter with Tet operators to the rDNA spacer, a transcriptionally silent locus [11], by transfecting KO1(1) cells with *Not* I-linearized pLew47. As shown in Fig. 3B, cells grown for 30 days without Tet exhibited an increase of CAT activity to constitutive levels after only 8 h exposure to Tet. However, the background of CAT activity even after growth for 30 days in the absence of Tet was significant, again suggesting a leaky regulation, perhaps due to a low level of expression of the Tet repressor in the GDH locus.

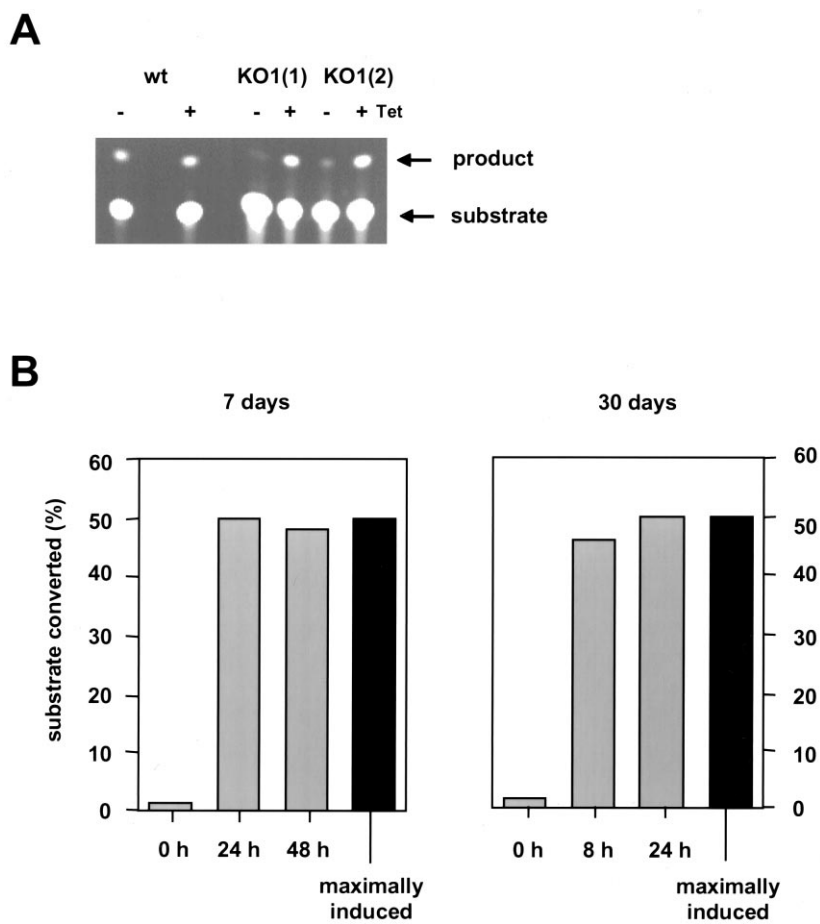

Fig. 3. Functional assay for Tet regulation in the single KO cell lines (Strategy I). (A) Transient CAT assays. Two TetR-HYGRO independent transfectants, KO1(1) and KO1(2), were tested for the ability of TetR to mediate efficient repression. After electroporation with supercoiled pLew47 DNA (Fig. 1), cells were split into two flasks and Tet was added to one at a concentration of 5 μg/ml. After 24 h, protein extracts were obtained and CAT assays performed as described in Section 2. (B) Regulation of chromosomally-integrated CAT. The single KO cell line, KO1(1), was further characterized for Tet regulation by integrating the CAT gene under the control of a PARP promoter with Tet operators in the rDNA 18S spacer locus. Cells were transfected with *Not*I-linearized pLew47 DNA and selected in the presence of 1 μg/ml of phleomycin and 5 μg/ ml of Tet. After selection, both antibiotics were withdrawn and cells were further incubated for 7 or 30 days. At time 0 h, Tet was added at a final concentration of 100 ng/ml and samples were collected at the times indicated. The maximally induced value (black bars) refers to the cells maintained always in the presence of 5 μg/ml of Tet.

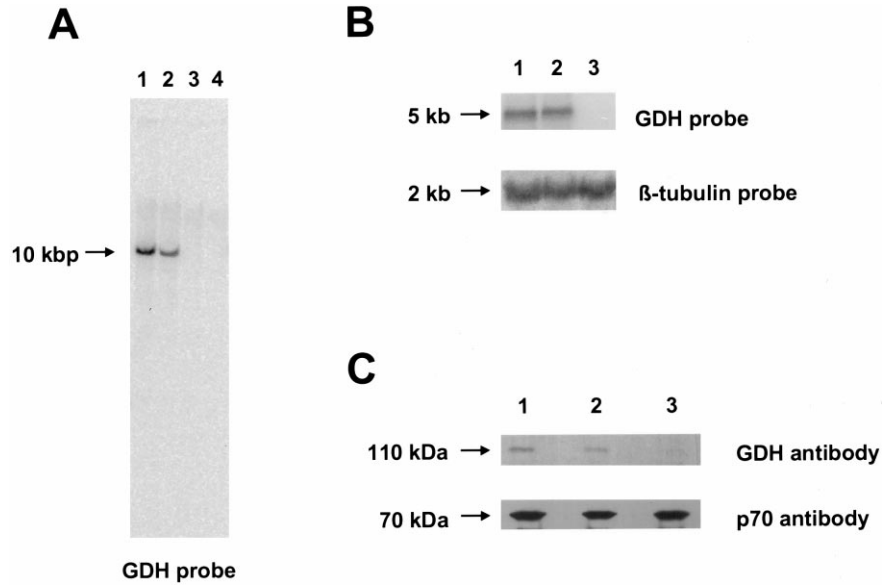

Fig. 4. Evidence for GDH double knockouts. (A) Southern blot performed with 10 μg of *Nhe*I-digested DNA from wild-type cells (lane 1), single KO (lane 2) and double KO cells (lane 3) obtained following Strategy I, and double KO cells obtained following Strategy II (lane 4). The entire GDH open-reading frame sequence was radiolabeled and used as a probe. B, Northern blot performed with 25 μg of total RNA from wild-type (1), and single (2) or double knockout (3) cell lines obtained from Strategy I. The entire GDH open-reading frame was radiolabeled and used as a probe. The filter was stripped and probed with a radiolabeled fragment of the β-tubulin gene (from plasmid pHD360 [12]) to confirm equal loading of RNA in all lanes. (C) Immunoblot of cell extracts obtained from the different cell lines (Strategy I) probed with an antibody against *L. tarentolae* GDH [1]. The filter was stripped and probed using an antibody against the mitochondrial p70 protein [32] as a gel loading control.

Replacement of the second GDH allele with NEO was performed in the KO1(1) cell line. These cells were electroporated with the pKO2b cassette (Fig. 1) and selected in the presence of 2.5 μg/ml of G418 (Gibco, BRL). Southern blot evidence for the loss of both GDH loci is shown in Fig. 4. The loss of the GDH mRNA and the protein product in the double KO line is also shown in this figure. The double KO cells were viable and had no apparent phenotype in culture at 37°C. Attempts to transform the cells into procyclic forms at 27°C were unsuccessful, but the wild type cells used for the transfections also failed to undergo differentiation, possibly as a result of the selection scheme employed to increase the yield of cells in the 37°C culture. No attempt was made to insert an ectopic copy of GDH since the Tet regulatory system in this line was leaky.

3.3. Strategy II: replacement of the first allele of GDH with the T7 RNA polymerase and replacement of the second allele with TetR under the control of a T7 promoter

Due to the incomplete repression of the reporter CAT activity in the above cell line, we decided to obtain a higher level of TetR expression by using a T7 promoter. In this strategy, the first allele of GDH was replaced by the T7 RNA polymerase and HYG genes. Wild-type cells were electroporated with the pT7KO1c cassette (Fig. 1) and selected in the presence of 1 μg/ml of hygromycin.

The second allele was then replaced by a TetR-NEO cassette under control of a mutated 10% efficient T7 promoter [14] by transfecting single KO cells with the pKO2e cassette (Fig. 1). Cells were selected in the presence of 2.5 μg/ml of

G418. Evidence for the loss of both GDH alleles was obtained (Fig. 4). The double KO cells were viable and showed no obvious phenotype.

A transient assay was performed for the presence of functional T7 RNA polymerase in the single KO cells. The cells were transfected with supercoiled pT7CAT1 (Fig. 1), that contains a CAT reporter gene under the control of a T7 promoter. As shown in Fig. 5A, the single KO cells expressed a high level of CAT activity, indicating the presence of functional T7 RNA polymerase.

Another transient functional assay was performed for Tet regulation in the single and double KO cells. Both cell lines were electroporated with supercoiled pLew47. As shown in Fig. 5B, the single KO cells exhibited no regulation of CAT activity by Tet, whereas the double KO cells, which contained the promoter-driven TetR cassette, showed a strong regulation. The background level of CAT expression in the double KO cells in the absence of Tet was undetectable by this assay, suggesting a tight regulation of CAT expression.

This strain is now available for insertion of an ectopic copy of the GDH gene under control of the PARP promoter with tet operators and this work is in progress.

3.4. Primer extension assays for RNA editing in GDH knockout mutants

The steady state abundances of three edited mRNAs were assayed in the single and double KO cell lines (Strategy I) by primer extension analysis. Edited and unedited primers localized within the editing domains were used to assay editing of the pan-edited RPS12 and A6 mRNAs. A primer localized downstream of the editing domain was used to assay editing of the 5′ edited MURF2 mRNA; in this assay both the edited and unedited extension products could be seen in one lane. Primer extensions of the never edited maxicircle-transcribed ND5 mRNA and the nuclear transcribed calmodulin mRNA served as controls. The primer extensions corresponding to the edited versions of RPS12 (Fig. 6A) and A6 mRNAs (Fig. 6B) showed some heterogeneity at the 5′ end, in agreement with previous findings made by sequencing cDNAs [21,22]. Two major bands were observed in the unedited A6 extension besides the expected one, what might also reflect heterogeneity in the 5′ end. MURF2 extensions (Fig. 6C) yielded two strong stops whose sizes correspond to the ones described for the unedited and the edited versions of this mRNA. Several extra bands were also detected that may corre-

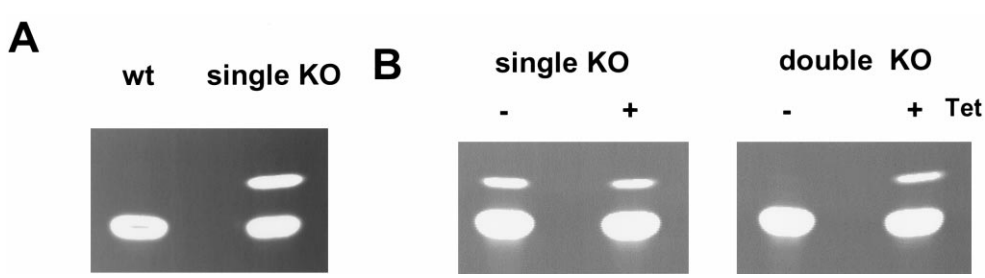

Fig. 5. Transient CAT assays for Strategy II knockouts. (A) Functional assay for T7 RNA polymerase activity. Wild-type or hygromycin-resistant single KO cell lines were transfected with supercoiled pT7CAT1 (Fig. 1). After 24 h, cell extracts were obtained and CAT assays performed as described in Section 2. (B) Functional assay for Tet regulation. A double cell line expressing both T7 RNA polymerase and TetR under the control of a mutated T7 promoter was assayed for Tet induction. A single KO cell line expressing only the polymerase was included as a control. Both cell lines were electroporated with supercoiled pLew47 (Fig. 1). After electroporation, cells were split in two flasks and Tet was added to one of them at a concentration of 5 μg/ml. CAT assays were performed after 24 h as described in Section 2.

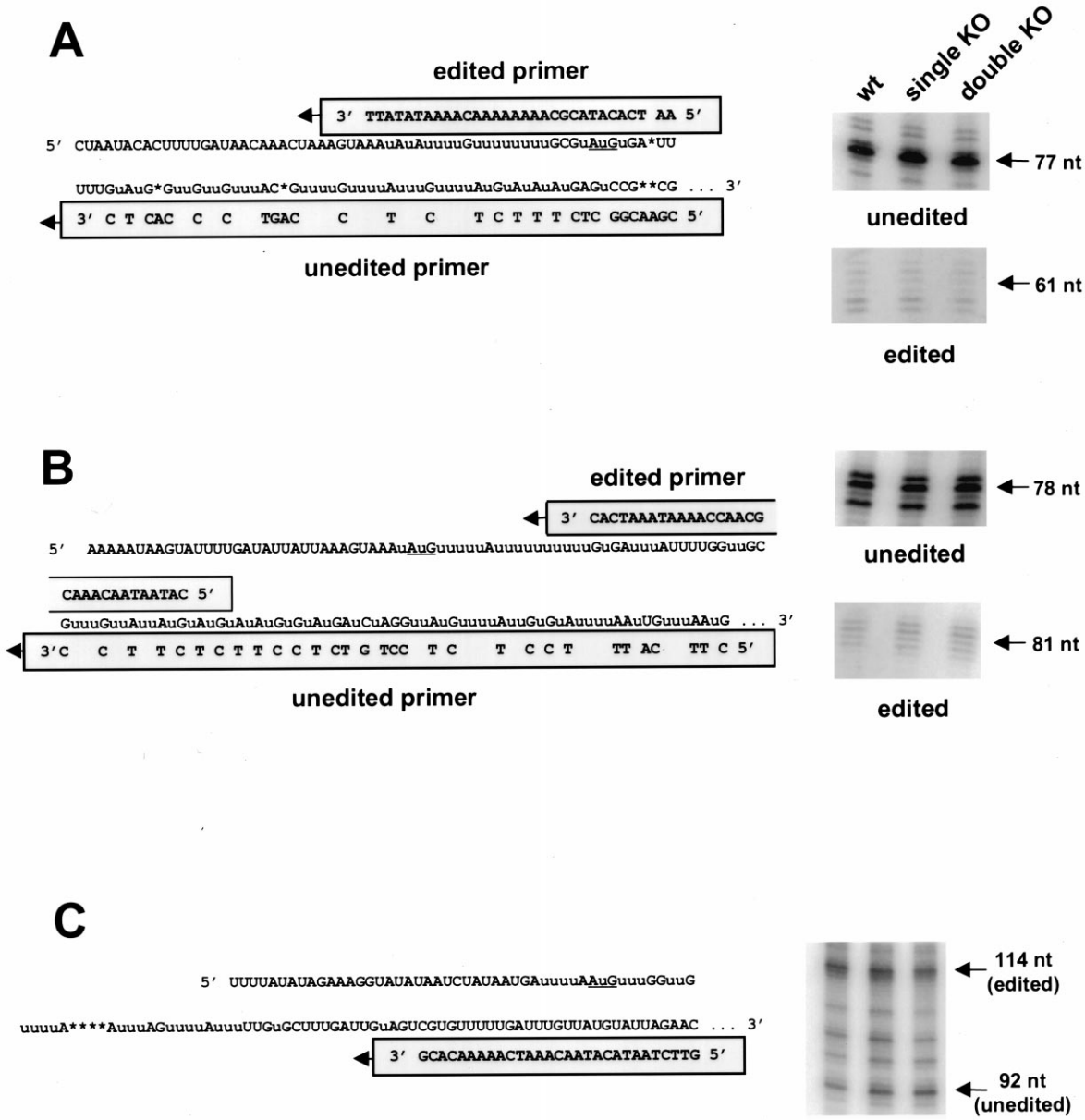

Fig. 6. Primer extension assays for RNA editing in the GDH mutants. The pan-edited mRNAs RPS12 (A) and A6 (B) were assayed with two different oligodeoxynucleotides, one specific for the 5′ unedited mRNA and the other specific for the edited version. MURF2 mRNA (C) was assayed using only one oligodeoxynucleotide that hybridized downstream of the editing domain, thereby extending the unedited, fully-edited and partially-edited species. The mitochondrial never-edited mRNA ND5 (D) and the cytoplasmic calmodulin mRNA (E) were used as controls. The sequences corresponding to the oligodeoxynucleotides are boxed and the arrows indicate the direction of extension by reverse-transcriptase. Added uridines are indicated in lowercase. The position of genomically encoded uridines deleted from the transcripts are marked with asterisks. The initiation codons are underlined. SAS refers to the splice-acceptor site in the calmodulin mRNA.

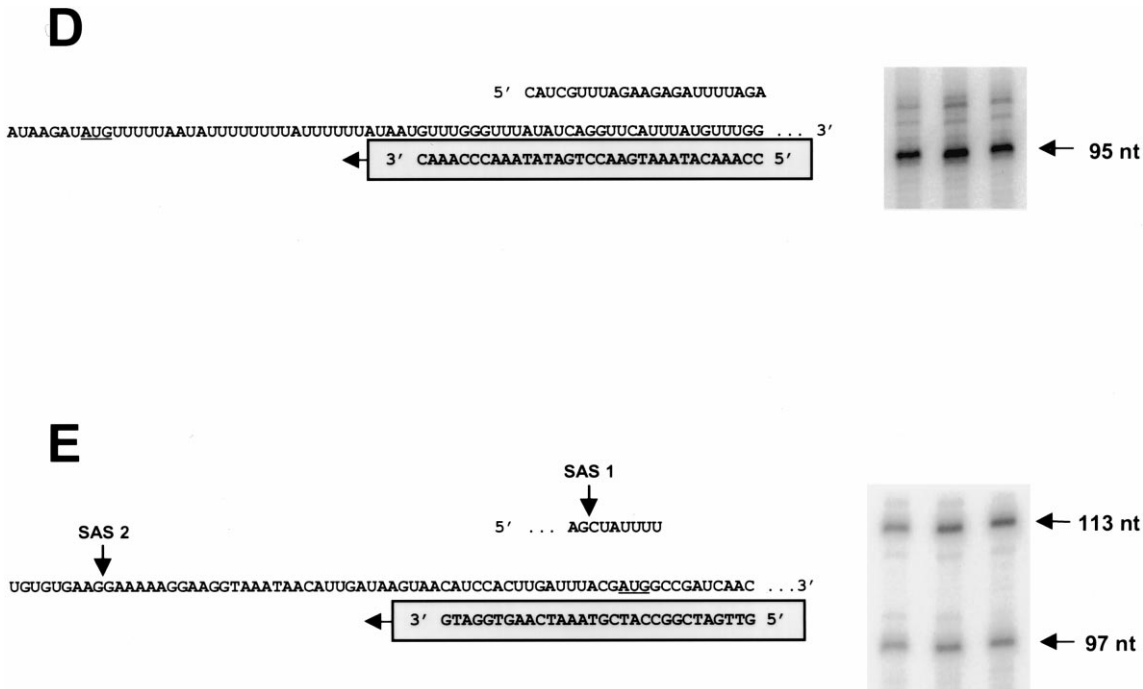

Fig. 6. (*Continued*)

spond to partially-edited species. ND5 extension (Fig. 6D) yielded only one strong stop whose size was one nucleotide longer that the expected one [21]. Extensions performed with a different oligodeoxynucleotide mapped the 5′ end of this mRNA at the same position (data not shown). This discrepancy might reflect differences among *T. brucei* strains. The primer extension performed on calmodulin mRNA (Fig. 6E) yielded the expected products [23].

The primer extension assay indicates that the steady-state levels of edited and unedited mRNAs were approximately identical in the wt, single KO and double KO cells. There was no effect of the GDH gene knockout on the abundance of the unedited ND5 mRNA and the nuclear-encoded calmodulin mRNA.

4. Discussion

Glutamate dehydrogenase is a metabolic enzyme involved in degradation and biosynthesis of amino acids. Evidence for a specific interaction of the mitochondrial GDH in *L. tarentolae* with the 3′ oligo[U] tail of gRNAs, apparently within the dinucleotide pocket of the enzyme, was previously presented [1]. In the experiments reported in this paper, we analyzed the phenotype of GDH null mutants of *T. brucei* bloodstream trypanosomes in culture. We selected cultured bloodstream trypanosomes of *T. brucei* for these experiments because editing occurs in several mRNAs [2], but is dispensable as shown by the viability of dyskinetoplastic mutants lacking detectable kineto plast DNA in this stage of the life cycle [24]. In the bloodstream stage of the life cycle, *T. brucei* cells live glycolytically and do not exhibit aerobic respiration and other mitochondrial functions [25]. On the other hand, *Leishmania* sp. appear to require aerobic respiration throughout their life cycle [26], and procyclic *T. brucei* also require aerobic respiration for survival [25], making it likely that null mutants of genes encoding proteins involved in editing or mitochondrial metabolic pathways would not be viable in this

stage of the life cycle. Of course, it is always possible that an editing gene could play multiple roles in cell metabolism and that a loss of this gene in bloodstream trypanosomes could also be lethal for reasons unrelated to loss of editing of mRNAs.

We found that GDH null mutants of *T. brucei* bloodstream cells in culture were viable and exhibited no changes in the relative abundance of edited mRNAs for three maxicircle cryptogenes, RPS12, A6 and MURF2. This negative evidence suggests that the binding of gRNA by GDH may not play a functional role in the mechanism of RNA editing, at least in the bloodstream stage of the life cycle. However, a negative result with gene knockout experiments must always be qualified by the possibility of the existence of redundant genes, the protein products of which could complement the role of GDH in the editing reaction. In fact, a number of proteins have been identified in trypanosome mitochondrial extracts by UV crosslinking experiments which bind gRNA, and some of these appear to specifically bind the oligo[U] tail [27–31]. Therefore a definitive proof for the lack of a role of GDH in editing must await biochemical fractionation and reconstitution of the editing machinery.

The possibility that GDH may play a role in the regulation of editing of those genes whose transcripts are specifically edited in the procyclic stage of the life cycle has also not been eliminated.

These experiments were also designed to establish a general protocol for double gene knockouts and conditional expression of genes presumably involved in editing. Two strategies were examined and the optimal one involved a replacement of the first allele with T7 RNA polymerase and the replacement of the second allele with TetR controlled by a mutated T7 promoter [14]. Using this procedure for the GDH knockouts in *T. brucei*, we examined the regulation of expression of a reporter gene with Tet using transient assays. There was a tight regulation of expression of the reporter gene in these cells, suggesting that this protocol may provide a general method for conditional expression of genes for mitochondrial proteins not required for viability during the bloodstream stage of the trypanosome life cycle. In cases where the null mutant cells are viable, the gene being tested would then be inserted into the genome in a silent locus under control of the PARP promoter with Tet operators. If the null mutants are not viable, then the ectopic copy would have to be inserted before the second KO is performed, followed by disruption of the second allele in the presence of Tet. This approach should permit establishment of conditional null mutants for genes involved in either essential or not essential cellular functions, such as RNA editing.

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