Heterologous Expression of the Bifunctional Thymidylate Synthase-Dihydrofolate Reductase from *Leishmania major*†

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ABSTRACT: The bifunctional thymidylate synthase–dihydrofolate reductase (TS–DHFR) of *Leishmania major* has been cloned and expressed in *Escherichia coli* and *Saccharomyces cerevisiae*. The strategy involved placing the entire 1560-bp coding sequence into a parent cloning plasmid that was designed to permit introduction of unique restriction sites at the 5′- and 3′-ends. In this manner, the entire coding sequence could be easily subcloned into a variety of expression vectors. High levels of TS–DHFR gene expression were driven by tac, pL and T7 RNA pol promoters in *E. coli*, and the GAPDH–ADH-2 promoter in *S. cerevisiae*. *L. major* TS–DHFR also complemented TS deficiency in *E. coli*. In *E. coli*, the protein accumulated to very high levels, but most was present as inactive inclusion bodies. Nevertheless, substantial amounts were soluble; up to 2% of the soluble protein was catalytically active TS–DHFR. In the yeast systems, essentially all of the bifunctional protein was soluble and catalytically active, and crude extracts contained about 100-fold more enzyme than do extracts from wild-type *L. major*. The expressed TS–DHFR from yeast and *E. coli* was purified to homogeneity by methotrexate–Sepharose affinity chromatography. About 8.5 mg of homogeneous, catalytically active protein is obtained from a 1-L culture of yeast, and 1.5 mg was obtained from 1 L of *E. coli* culture. A 200-L fermentation of the yeast expression system yielded a crude extract containing over 4 g of TS–DHFR. The TS–DHFR from yeast was blocked at the N terminus, as is the enzyme from *Leishmania*; in *E. coli*, the protein showed an N-terminal serine, which corresponds to amino acid 2 of the coding sequence. With the availability of recombinant high-expression systems, structure/function and mutagenesis studies of the bifunctional TS–DHFR of *Leishmania* are now possible.

Thymidylate synthase (TS;[1] EC 2.1.1.45) and dihydrofolate reductase (DHFR; EC 1.5.1.3) catalyze sequential reactions in the de novo synthesis of dTMP. TS and DHFR exist as distinct and readily separable proteins in bacteriophage, bacteria, mammalian viruses, yeast, and vertebrates [see Blakley (1984) and Santi and Danenberg (1984)]. TS is a highly homologous dimer of identical subunits of about 35 kDa, and DHFR is a monomer of about 20 kDa. In contrast, TS and DHFR exist as a bifunctional protein in protozoa (Ferone & Roland, 1980; Garrett et al., 1984); depending on species, the apparent sizes usually range from 110 to 140 kDa, with subunit sizes of 55–70 kDa.

The TS–DHFR from the protozoan parasite *Leishmania major* has been purified and partially characterized (Meek et al., 1985), and its sequence has been predicted from the DNA sequence (Grumont et al., 1986; Beverley et al., 1986). Each 60-kDa subunit consists of two domains, with DHFR at the amino terminus and TS at the carboxy terminus, separated by a junction peptide. The domains are related in size and structure to their monofunctional counterparts in other organisms and in the native protein show structural and functional communications with one other. We have suggested that the junction peptide may vary in size in certain protozoa (Garrett et al., 1984).

We have been interested in structure/function aspects of TS and DHFR, and the unique properties of the bifunctional protein have made the TS–DHFR of protozoa a focus of attention in our laboratory. Thus far, the major sources of this protein have been antifolate-resistant strains of *L. major*, which produce about 10-fold more TS–DHFR than wild-type organisms (Coderre et al., 1983; Garvey & Santi, 1985). There were two compelling reasons for us to develop heterologous expression systems for the *Leishmania* TS–DHFR. First, the low amounts of protein obtainable from protozoan sources place severe limitations on the type and scope of studies we can consider performing. Even with the antifolate-resistant overproducing strains of *L. major*, we can only obtain about 2 mg of protein/10 L of culture of potential pathogenic organisms. Second, since transformation systems in *Leishmania* are not available, heterologous expression systems are necessary for any anticipated mutagenesis studies.

In the present paper, we describe highly effective systems for the expression of catalyticallly active *L. major* TS–DHFR in both bacteria and yeast. This is the first example of expression of a catalytically active enzyme from *Trypanosoma*

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[1] Abbreviations: TS–DHFR, thymidylate synthase–dihydrofolate reductase; H_2folate, 7,8-dihydrofolate; H_4folate, tetrahydrofolate; CH_2-H_4folate, 5,10-methylenetetrahydrofolate; MTX, methotrexate; ON, oligonucleotide; RP-HPLC, reverse-phase HPLC; TFA, trifluoroacetic acid; ACN, acetonitrile; DTT, dithiothreitol; BME, β-mercaptoethanol; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; YP, 1% bacto-yeast extract, 2% bactopeptone; YPD, YP with 2% dextrose; SD-leucine, 0.67% bacto-yeast nitrogen base without amino acids, 2% glucose, constituted with essential amino acids except leucine; IPTG, isopropyl β-D-thiogalactoside; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; IEF, isoelectric focusing. All other abbreviations are those recommended by IUPAC.

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in heterologous systems. We can now obtain the large amounts of the bifunctional protein needed for structure/function studies, and the systems are well suited for site-specific mutagenesis studies.

Materials and Methods

Materials. Yeast 2μ plasmid PC1/1 (Travis et al., 1985) and shuttle vector pBS100 (Barr et al., 1987) were gifts from Dr. P. Barr (Chiron Corp.). Expression vector pAR3038 and *Escherichia coli* K12 strain BL21 (Studier & Moffat, 1986) were gifts from Dr. W. Studier (Brookhaven National Laboratories). Expression vectors pMG27N-S and pOTS-*Nco*I, and *E. coli* K12 strains MM294cI$^+$, N5151, and AR120 (Shatzman & Rosenberg, 1986) were gifts from Dr. M. Rosenberg (Smith Kline and Beckman Laboratories). *E. coli* K12 strains x2913 (ΔthyA572) and D1210 (lacI^Q) were gifts from Drs. R. Thompson (Glasgow University) and P. Greene (UCSF), respectively, and *Saccharomyces cerevisiae* strain AB103.1 was a gift from Dr. R. T. Raines (UCSF). Plasmids pK3B and pK7 containing inserts of *L major* genomic DNA were those previously reported (Washtien et al., 1985). Oligonucleotide synthesis and peptide sequencing were performed at the UCSF Biomolecular Resource Center. The sequences of the oligonucleotide duplexes used are listed in Table I. MTX-Sepharose CL-6B, purified *L. major* TS-DHFR (Meek et al., 1985), and H_2folate (Futterman, 1957) have been described. All other materials were as described (Meek et al., 1985) or the purest commercially available.

DNA Manipulations. The general methods for ligation (Maniatis et al., 1982), transformation of *E. coli* (Hanahan, 1983) or *S. cerevisiae* by the zymolyase procedure (Beggs, 1978), and rapid screening of clones by restriction analysis (Fromm & Berg, 1983) were performed as described. Recovery of electrophoresed DNA from low-melting agarose (SeaPlaque, FMC) was performed according to the method of Wieslander (1979). Adapters were ligated to purified restriction fragments and vectors by modifications of reported methods (Maniatis et al., 1982; Wieslander, 1979). Equal amounts of complementary strands of the adapters (0.01 OD/μL) were treated at 65 °C for 5 min, followed by reassociation at 37 °C for 2 h and at 4 °C for 4–8 h. For cassette ligations, annealed adapters were ligated to endonuclease-digested vectors at molar ratios of 10:1 and 100:1 (50 ng of vector in 10 μL). The modified vectors were purified by electrophoresis on low-melting agarose (Wieslander, 1979) and transformed into the appropriate *E. coli* host. For fragment ligations used in the construction of the yeast shuttle vector pS2, annealed adapters were ligated to the appropriate restriction fragment at molar ratios of 10:1, 50:1, and 100:1 (50 ng of vector of 10 μL). The restriction fragment containing the ligated adapter was purified by low-melting agarose electrophoresis, ligated into shuttle vector pBS100, and transformed into *E. coli*. The *Bam*HI expression cassette of shuttle vector pS2 was then excised and cloned into the yeast 2μ vector PC1/1. Authenticities of the gene constructions were initially confirmed by restriction analysis at sites created by the introduced adapter and at a diagnostic restriction sites (viz, *Pvu*II, *Bgl*II, *Xho*I, and *Pst*I) within the TS-DHFR gene (Grumont et al., 1986). Constructs pS1, pS2, pS1N, and pS1B were subcloned into m13mp18/19, propagated in JM101, and sequenced through the introduced adapters and ligation sites by the dideoxy method (Sanger et al., 1977).

Expression constructs were transformed into the appropriate hosts. pE1 (pKK223.3) was cloned into JM105 (lacI^Q); pE2 (pAR3038) was originally cloned and propagated in HB101 and subsequently cloned into expression host BL21 (Studier & Moffat, 1986); pE3a and pE3b (pMG27N-S and pOTS-*Nco*I), originally cloned and expanded in MM294cI$^+$, were cloned into expression hosts N5151 and AR120, respectively (Shatzman & Rosenberg, 1986). Yeast expression vectors pE4a and pE4b (PC1/1; Travis et al., 1985) were originally cloned and propagated in *E. coli* HB101 and then transformed into *S. cerevisiae* strain AB103.1 (Beggs, 1978).

Growth Conditions. Unless otherwise specified, bacterial clones were grown in LB containing 50 μg/mL ampicillin. Yeast clones were grown in YPD supplemented with 2% glucose. Low-density cultures were stored as glycerol stocks at −70 °C. For evaluation of expression products, bacterial cultures were initiated by 100-fold dilution of the glycerol stocks into media that, in the case of pE2, was supplemented with 0.4% glucose. pE1, pE2, and pE3b cultures were grown at 37 °C to OD_{600} = 0.6, 1, and 0.8, respectively. pE1 and pE2 cultures were then induced by the addition of 5 and 0.5 mM IPTG, respectively, and pE3b was induced with 5 mM nalidixic acid; cultures were grown for an additional 3 h at 37 °C (OD_{600} ~2). Expression vector pE3a, harbored in *E. coli* K12 strain N5151 which contains the c1857 temperature-sensitive lambda repressor, was grown at 32 °C until a cell density of OD_{600} = 0.8 was reached, then at 42 °C for 2.5 h (OD_{600} = 1.5; Shatzman & Rosenberg, 1986). Yeast harboring pE4a and pE4b were grown overnight to OD_{600} = 0.7 in SD-leucine selective media containing 8% glucose at 30 °C. Cultures were diluted 1:500 in YP supplemented with 1% glucose and grown approximately 2 days to stationary phase (OD_{600} ~8).

Preparation of Cell Extracts. Bacterial cell extracts were prepared by sonication. Yeast cells were processed by zymolyase/freeze thaw (Taylor et al., 1982) for small-scale or glass-bead disruption for large-scale preparations. All steps were performed at 0–4 °C. For bacterial cultures, harvested cells were suspended in about 2 volumes of 50 mM TES, pH 7.0, 75 mM BME, 1 mM EDTA, and the six-protease inhibitor mix described by Meek et al. (1985). Sonication was performed on ice using the micro (20 s) or standard $^3/_4$-in. probe (5 min) on the 50% duty pulse setting (Branson sonifier Model 350). Zymolyase/freeze thaw processing of small-scale yeast cultures was performed as described (Taylor et al., 1982). For large-scale yeast preparations, cells were disrupted in a "mini-beadbeater" (Biospec Products). The pellet was resuspended in 2 volumes of 50 mM TES, pH 7.0, 1 mM EDTA, and 75 mM BME containing a protease inhibitor mix (Meek et al., 1985); 1–2 volumes of 0.5-mm glass beads was added, and the suspension was beaten at 4 °C at 1-min intervals until the cells were disrupted (ca. 5 min) as determined by clearing of the suspension when a 10-μL aliquot was mixed with 1.0 mL of water. Homogenates were cleared by centrifugation (30000*g*, 1 h).

Protein Analysis. Protein was determined by the method of Read and Northcote (1981). Samples were dissolved in 0.1 volume of Laemmli buffer and analyzed on 12% SDS-PAGE (Laemmli, 1977) using Coomassie R250. Western blot analysis was performed as described (Burnette, 1981) using a 1:200 dilution of rabbit anti-*L. major* TS-DHFR sera (Garvey & Santi, 1985) affinity purified against *E. coli* lysate (Kemp et al., 1983). RP-HPLC of purified proteins was performed using a C_8 RP-300 (2.1 × 30 mm) column and an ABI 130 HPLC. About 1 μg of purified protein was applied to the column and eluted at 200 μL/min with a linear gradient of 0.1% TFA/water to 0.1% TFA/70% ACN over 45 min. Elution was monitored by UV absorption at 215 nm. Peptide sequencing was performed on an ABI 470A protein se-

quencer/120A PTH analyzer. To verify that the amino terminus of yeast TS–DHFR was blocked, about 500 pmol of TCA-precipitated protein was dissolved in 50 μL of 70% formic acid. An aliquot (10 μL) was applied directly to the sequencer for N-terminal analysis; the remainder was treated with 1 volume of 120 mg/mL CNBr in 70% formic acid for 24 h protected from light and applied to the sequencer to obtain N termini of the CNBr fragments. For horizontal isoelectric focusing (IEF), the gel solution [containing 20% glycerol, 5.4% polyacrylamide, 2.2% ampholines, pH 3.0–10.0 (Biolyte; BioRad Inc.), 0.2% TEMED, and 0.04% $(NH_4)_2S_2O_8$] was cast between two glass plates (80 × 100 × 0.75 mm). After the instrument was prefocused at 5 W for 30 min, about 1.0 μg of purified enzyme was focused for 1.5 h at 5 W. The gels were fixed in 5% sulfosalicylic acid/10% TCA for 1 h followed by 30% MeOH/10% AcOH for 30 min prior to staining with Coomassie R250. The pH gradient was determined by electrophoresis of IEF standards, p*I* 4.6–9.6.

TS and DHFR activity determinations and purifications of expressed TS–DHFR were performed essentially as described for the *Leishmania* enzyme (Meek et al., 1985).[2] Immediately after cell disruption, soluble extracts containing up to 500 mg of protein were applied to a 4-mL MTX–Sepharose CL-6B column (1.0 × 5 cm; ~1 μmol/mL) that had been equilibrated with 10 mM potassium phosphate, pH 7.0.[2,3] The extract was circulated through the column at 0.3 mL/min for 3–4 h at 4 °C until there was no further loss of enzyme activity in the circulant; generally, about 95% of the enzyme activity was bound to the gel. The column was washed with 10 mM potassium phosphate, pH 7.0, containing 1 M KCl, until the effluent was colorless (ca. about 8 volumes; 35 mL) and then with the buffer lacking KCl (ca. 5 volumes; 20 mL). Six milliliters (1.5 volumes) of 2 mM H_2folate in 50 mM TES, pH7.4, 5 mM DTT, and 1 mM EDTA was applied, simultaneously removing an equal amount of buffer from the outlet, and allowed to equilibrate for 20 min. TS–DHFR was eluted with the potassium phosphate buffer, collecting 1.0-mL fractions. Fractions containing TS–DHFR activities (1–6 mL) were pooled and concentrated to about 1 mL (Centricell; Polyscience), and H_2folate was removed by filtration through a Sephadex G-15 column (1 × 10 cm). For larger preparations, up to 50 mL of extract (2.2 g of total protein) was applied to a 50-mL MTX–Sepharose column (3 × 7 cm); washing, elution, concentration, and H_2folate removal were scaled up accordingly from the above procedure.

Inclusion Bodies. Cultures were washed once in LB, and then a suspension was sealed under a glass coverslip and visualized by phase-contrast microscopy (4000×). Inclusion bodies were isolated from the pellet of sonicated bacterial extracts by the method of Marston et al. (1984). The pellet was washed in 9 volumes of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 50 mM NaCl containing 0.5% Triton X-100/10 mM EDTA, and inclusion bodies were isolated by centrifugation (1000*g*, 10 min).

Complementation of TS-Deficient E. coli. pE1, pE3a, and pBR322 (as a control) were transformed (Hanahan, 1983) into the TS-deficient *E. coli* K12 strain x2913. Transformation mixtures were plated on duplicate minimal agar plates (Belfort et al., 1983) containing 50 μg/mL ampicillin, with or without 50 μg/mL thymine (Thy). Untransformed x2913 used in these experiments was plated on duplicate Thy± minimal plates not containing ampicillin to verify maintenance of the Thy⁻ phenotype.

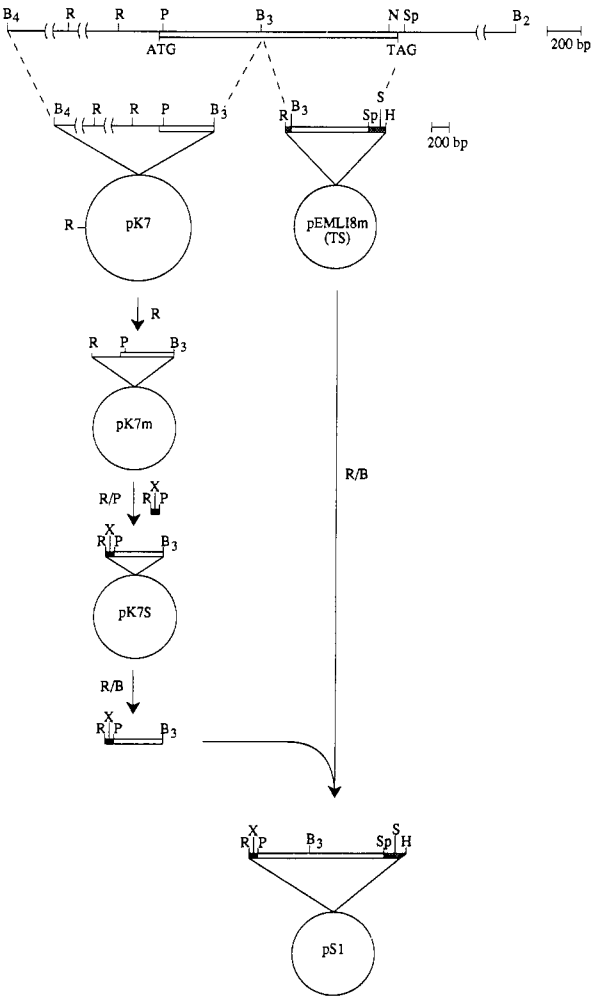

FIGURE 1: Construction of the parent cloning plasmid pS1 containing the TS–DHFR gene. The open bars represent coding sequence derived from genomic DNA (gDNA), and shaded bars are sequences derived from the synthetic oligonucleotide adapters ON1 (partial shading) and ON2 (solid shading) described in Table I. Restriction endonuclease sites are noted R for *Eco*RI, B for *Bgl*II, P for *Pvu*II, N for *Nci*I, Sp for *Sph*I, S for *Sal*I, H for *Hin*dIII, and X for *Xba*I. On the left, pK7 was reduced to pK7m by restriction digestion with *Eco*RI and ligation; the *Eco*RI–*Pvu*II fragment of pK7m was replaced with ON2, which contains a silent *Xba*I site, to give pK7s. On the right, pEMBL18m was prepared by inserting the *Bgl*II$_3$–*Sph*I TS fragment of gDNA from pK3B into a pEMBL18 vector with the modified polylinker ON1. The *Eco*RI–*Bgl*II$_3$ fragment from pK7s, containing DHFR, was excised and inserted into pEMBL18m to provide the parent plasmid pS1 containing the TS–DHFR coding sequence in its entirety.

RESULTS

The starting points for this study were the two *Bgl*II gDNA fragments (*Bgl*II$_4$–*Bgl*II$_3$ and *Bgl*II$_3$–*Bgl*II$_2$) from chromosome 4 (Garvey & Santi, 1986) of *L. major* that possess the 1560-bp coding sequence of TS–DHFR (Figure 1). pK7 contains the 7-kb *Bgl*II$_4$–*Bgl*II$_3$ fragment that has 6.4 kb of 5′ noncoding sequence and all of the DHFR domain; pK3B contains the 3.3-kb *Bgl*II$_3$–*Bgl*II$_2$ fragment that has the TS domain followed by 2.4 kb of 3′-untranslated sequence. We reduced the

[2] We have previously reported that the TS activity of TS–DHFR is extremely susceptible to proteolysis, both by endopeptidases and by carboxypeptidase (Garvey & Santi, 1985); carboxypeptidase products are inseparable from the unmodified TS–DHFR subunit by SDS–PAGE. Even purified preparations undergo preferential significant loss of TS activity upon storage (Meek et al., 1985); the reason for this instabilty is unknown.

[3] Initial affinity chromatography should be performed as soon and as rapidly as possible after cell disruption to avert proteolysis in the crude extract.

Table I: Synthetic Oligonucleotide Duplexes Used in Cloning the TS-DHFR

oligo	restriction sites	sequence[a]	position	vector
ON1	*Eco*RI/*Bgl*II *Sph*I/*Sal*I/*Hin*dIII	AATTCAGATCTGCATGCGTCGACA GTCTAGACGTACGCAGCTGTTCGA	polylinker	pEMBL18m
ON2	*Eco*RI/*Xba*I/*Pvu*II	AATTCATGTCTAGAGCAG GTACAGATCTCGTC	5′	pS1
ON3	*Eco*RI/*Nde*I/*Xba*I	AATTCATATGT GTATACAGATC	5′	pS1N
ON4	*Eco*RI/*Nae*I/*Bsp*MI/*Xba*I	AATTCACCTGCCGGCCATGT GTGGACGGCCGGTAGAGATC	5′	pS1B
ON5	*Nco*I/*Xba*I/*Pvu*II	CATGTCTAGAGCAG AGATCTCGTC	5′	pS2
ON6	*Nci*I/*Sal*I	CGGCGATCAAGATGGAGATGGCCGTATAG CCGCTAGTTCTACCTCTACCGGCATATCAGCT	3′	pS2

[a] Underlined areas represent TS-DHFR coding sequence.

size of each of these fragments and modified the 5′- and 3′-ends to permit facile introduction of unique cloning sites. In particular, we introduced a unique silent *Xba*I site at nucleotide 4 of the DHFR sequence. Concurrently, the polylinker of pEMBL18 was modified to contain restriction sites to facilitate sequential cloning steps (see below). We then reassembled the entire coding sequence in a parent cloning vector by joining the DHFR and TS fragments at their common $Bgl\text{II}_3$ sites. From this vector, the 5′ cloning sites were introduced by cassette insertions of oligonucleotides into the *Eco*RI–*Xba*I sites, and the TS–DHFR gene was moved in toto into various expression systems (Figure 2).

Cloning Vector pEMBL18m. The polylinker of pEMBL18 was replaced by the 24-bp synthetic ON1 containing *Eco*RI–*Bgl*II–*Sph*I–*Sal*I–*Hin*dIII (Table I). This adapter contains unique features relevant to the construction: (a) a *Bgl*II–*Sph*I/*Sal*I fragment that can be replaced by the TS coding sequence; (b) *Eco*RI–*Bgl*II sites for insertion of the DHFR sequence adjacent to and in frame with TS; (c) several other unique sites for alternate cloning approaches and excision of the entire coding sequence.

TS Domain. A 920-bp *Bgl*II–*Sph*I restriction fragment from genomic clone pK3B (Washtien et al., 1985) containing the entire carboxy-terminal TS domain of the TS–DHFR gene (amino acids 225–520; nucleotides 668–1563) and 35 bp of 3′ untranslated region was cloned into the *Bgl*II–*Sph*I sites of pEMBL18m (Figure 1).

DHFR Domain. The 5′ portion of the TS–DHFR gene, containing the DHFR domain, was obtained by reduction of the genomic clone pK7 such that the modified plasmid (pK7m) contained amino acids 1–224 of the TS–DHFR gene as well as 243 bp of 5′ untranslated region (Figure 1). pK7 contains the 7-kb $Bgl\text{II}_4$–$Bgl\text{II}_3$ genomic sequence in the orientation where the 0.9-kb 5′ *Eco*RI–$Bgl\text{II}_3$ fragment, containing the DHFR domain, is furthest from the *Eco*RI site of the vector. *Eco*RI digestion of pK7 provided three fragments. The 3.3-kb *Eco*RI fragment containing the 0.9-kb 5′ *Eco*RI–$Bgl\text{II}_3$ *Leishmania* fragment (with the DHFR sequence) was isolated, circularized by ligation, and transformed into D1210 to give pK7m. Digestion of pK7m with *Eco*RI and *Pvu*II removed the 243 bp of the 5′ untranslated region as well as the first 13 bp of the coding region. This sequence was replaced with a 18/14-bp *Eco*RI–*Pvu*II adapter (ON2) to give pK7s. This reconstructed the first five amino acids of TS–DHFR, created a unique silent *Xba*I site one base downstream of the initiation methionine, and placed the *Eco*RI site five bases upstream of the start codon (Figure 1).

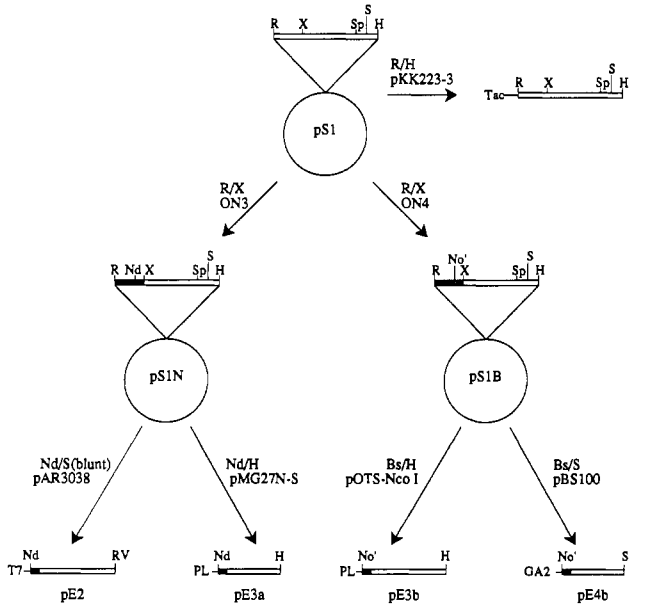

FIGURE 2: Strategy for the transfer of the TS–DHFR coding cassette from pS1 into expression vectors. Restriction sites are denoted Bp for *Bsp*MI, R for *Eco*RI, H for *Hin*dIII, S for *Sal*I, Sp for *Sph*I, V for *Eco*RV, Nd for *Nde*I, and No′ for *Nco*I four-base overhang. The T7 RNA pol promoter is denoted T7 and the GAPDH–ADH-2 promoter as GA2. The 5′ *Eco*RI–*Xba*I fragment of parent cloning plasmid pS1 was replaced with oligonucleotides ON3 (solid shading) or ON4 (partial shading) containing internal restriction sites designed for generating unique 5′ *Nde*I or *Nco*I start codon cloning sites; the *Nco*I overhang is generated by cleavage with *Bsp*MI that recognizes a site four bases upstream.[4] The TS–DHFR coding sequence is mobilized and inserted into expression vectors containing the 5′ *Nde*I (pS1N) or *Nco*I (pS1B) and 3′ *Hin*dIII, *Sal*I, or *Eco*RV sites.

TS–DHFR Parent Plasmid pS1. The parent cloning plasmid pS1 was obtained by inserting the *Eco*RI–*Bgl*II fragment of pK7s into the corresponding sites of pEMBL18m, contiguous with the TS domain (Figure 1). The entire TS–DHFR coding sequence was thus reconstructed, containing the silent *Xba*I site that was previously introduced adjacent to the start codon. This allowed insertion of *Eco*RI–*Xba*I synthetic adapters into pS1 that contain the initiation codon and any upstream restriction site(s) required for cloning into expression vectors (Figure 2).

Construction of Expression Vectors. Unique cloning sites placed upstream of the start codons and 3′ to the coding se-

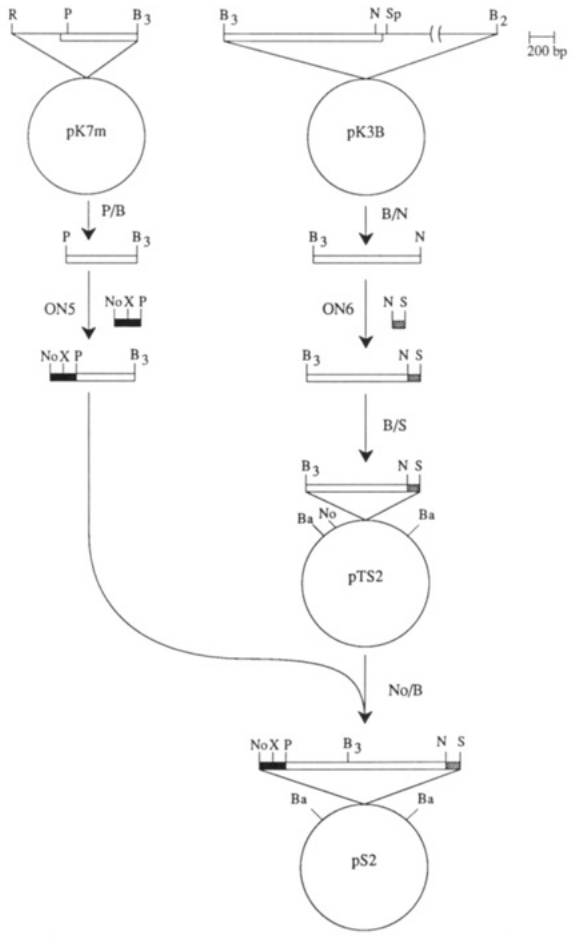

FIGURE 3: Construction of the yeast expression cassette in pS2 containing the TS–DHFR coding sequence. The open bars represent coding sequence derived from gDNA, and shaded bars are from synthetic oligonucleotides ON5 (solid shading) and ON6 (partial shading). Restriction sites are denoted Ba for *Bam*HI, B for *Bgl*II, R for *Eco*RI, P for *Pvu*II, N for *Nci*I, Sp for *Sph*I, X for *Xba*I, S for *Sal*I, and No for *Nco*I. On the right, the TS genomic fragment *Bgl*II$_3$–*Nci*I from pK3B was ligated to ON6 and inserted into the shuttle vector pBS100 to give pTS2. On the left, the DHFR genomic fragment *Pvu*II–*Bgl*II$_3$ from pK7m was ligated to ON5; the resultant *Nco*I–*Bgl*II fragment was introduced into the *Nco*I–*Bgl*II sites of pTS2 to give pS2 containing the TS–DHFR coding sequence within the *Bam*HI expression cassette of pBS100.

quence in pS1 permitted excision of the entire coding sequence. In this manner, the coding sequence was moved into optimized positions of a number of common expression vectors (Figure 2). Insertion of the *Eco*RI–*Hin*dIII fragment of pS1 into pKK223.3 to give plasmid pE1 placed the TS–DHFR start codon 10 bases downstream of the ribosome binding site, under the transcriptional control of the tac promoter. Replacement of the *Eco*RI–*Xba*I fragment of pS1 with a synthetic adapter (ON3) containing a unique *Nde*I site at the initiation methionine gave pS1N and permitted excision of the gene with an *Nde*I site at the start codon. The TS–DHFR gene was excised from pS1N and inserted as a *Nde*I–*Hin*dIII fragment into the *Nde*I–*Hin*dIII sites of pL vector pMG27N-S to give pE3a. In addition, the 3′ *Sal*I site of pS1N was digested and made blunt-end with T4 DNA polymerase. Subsequent digestion with *Nde*I released an *Nde*I/blunt-end fragment that was inserted into the *Nde*I–*Eco*RV sites of T7 RNA pol vector pAR3038 to give pE2. The *Eco*RI–*Xba*I region of pS1 was also replaced by an adapter containing *Eco*RI–*Bsp*MI–*Xba*I (ON4) to give pS1B; ON4 has the *Nco*I sequence 5′-CATG four bases downstream from the *Bsp*MI site and a *Nae*I site adjacent to *Eco*RI for additional flexibility.[4] Cleavage of

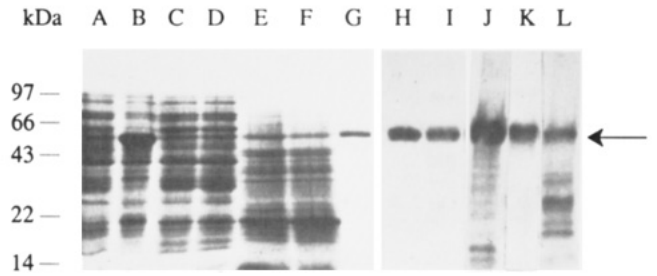

FIGURE 4: SDS–PAGE analysis of expression products in total cell extracts. Arrow indicates TS–DHFR. (Lanes A–G) Stained with Coomassie-R250; (lane A) pE1 (tac promoter); (lane B) pE2 (T7 RNA pol promoter); (lane C) pE3a (pL promoter, heat inducible); (lane D) pE3b (pL promoter, nalidixic acid inducible); (lane E) pE4a (GAPDH–ADH-2 promoter from pS2); (lane F) pE4b (GAPDH–ADH-2 promoter from pS1B); (lane G) TS–DHFR purified from CB3717-resistant *L. major* (0.5 μg). (Lanes H–L) Western blot analysis of *E. coli* and *S. cerevisiae* expression products; (lane H) purified TS–DHFR from CB3717-resistant *L. major* (0.5 μg); (lane I) pE1 (tac promoter); (lane J) pE2 (T7 RNA pol promoter); (lane K) pE3a (pL promoter); (lane L) pE4a-transformed *S. cerevisiae* (GAPDH–ADH-2 promoter).

pS1B with *Bsp*MI and *Hin*dIII provided a *Nco*I overhang at the 5′-end that enabled direct insertion of the TS–DHFR gene into the *Nco*I–*Hin*dIII sites of the pL vector pOTS-*Nco*T to give pE3b. Likewise, cleavage with *Bsp*MI–*Sal*I permitted cloning of the coding sequence into the *Nco*I–*Sal*I sites of yeast shuttle vector pBS100 to give pS3. The 3665-bp *Bam*HI expression cassette from pS3 containing the TS–DHFR gene flanked by the fused GAPDH–ADH-2 promoter and GAPDH transcriptional terminator was then cloned into the yeast 2μ vector PC1/1 giving pE4b.

Alternate Yeast Vector pE4a. An alternate vector was constructed for expression in yeast. Plasmid pS2 (Figure 3) was made by ligation of the 855-bp *Bgl*II$_3$–*Nci*I fragment from pK3B (which codes for all but the final 10 amino acids of the TS domain) with a 32/29-bp *Nci*I–*Sal*I adapter (ON6) containing the excised coding sequence and a stop codon. The ligated fragment was purified and cloned into the *Bgl*II–*Sal*I sites of the yeast shuttle vector pBS100 to give pTS2, containing the TS domain. The *Pvu*II–*Bgl*II fragment from pK7m was ligated to a 14/10-bp *Nco*I–*Xba*I–*Pvu*II adapter (ON5) containing the 13 bases of the 5′-end of the gene, thus reconstructing the DHFR domain. This fragment was inserted into the *Nco*I–*Bgl*II sites of pTS2 to give the entire coding sequence in the shuttle vector pS2 (Figure 3). The 3630-bp *Bam*HI expression cassette from pS2 was then cloned into the yeast 2μ vector PC1/1 giving pE4a.

Expression. Expression plasmids containing the TS–DHFR gene were transformed into *E. coli* K12 or *S. cerevisiae* hosts, and the authenticity of the plasmid constructs was again verified by restriction analysis. Cultures were grown under conditions appropriate for induction of expression; harvested cells were solubilized in Laemmli buffer and protein analyzed by SDS–PAGE. As shown in Figure 4 (lanes A–G), each of the induced expression systems shows an intensely staining protein that has M_r ~60 000 and comigrates with authentic *L. major* TS–DHFR. The expressed products react with rabbit antisera raised against purified *L. major* TS–DHFR (Figure 4, lanes H–L). Lanes J and L (pE2 and pE4a, re-

[4] *Bsp*MI restriction endonuclease cleaves four bases downstream of its recognition sequence, leaving a four-base overhang, on the 5′-end. Since *Bsp*MI has no specificity for the sequence between its binding and cleavage sites, this permits construction of any four-base overhang needed for ligation. It is particularly useful to produce a four-base overhang at the initiation codon for cloning into *Nco*I expression vectors.

Table II: Expression of *Leishmania* TS–DHFR

organism	plasmid	vector	promoter (induction)	% of total protein	specific act. (unit/mg) DHFR	TS	DHFR/TS
E. coli							
JM105	pE1	pKK223.3	tac (IPTG)	7	322	48	6.7
X2913(-TS)	pE1	pKK223.3	tac (none)		195	22	8.9
BL21	pE2	pAR3038	T7 pol (IPTG)	40	247	47	5.3
N5151	pE3b	pMG27N-S	pL (heat)	8	136	14	9.7
x2913 (-TS)	pE3b	pMG27N-S	pL (none)		75	7.7	9.7
S. cerevisiae							
wild type[a]						0.1	
AB103.1	pE4a	PC1/1	GAPDH–ADH-2	7	1885	175	10.8
AB103.1	pE4b	PC1/1	GAPDH–ADH-2 (-C source)	7	179	11	16.3
Leishmania[b]							
wild type				0.1	20	3	6.7
resistant				1	180	40	4.5

[a] Data from Taylor et al. (1982). [b] Data from Meek et al. (1985).

spectively) show other minor bands not seen in the purified preparation from *L. major* (lane H), which probably represent degradation products since TS–DHFR is quite susceptible to proteolysis (Garvey & Santi, 1985).

Leishmania TS–DHFR is expressed at varying levels in the systems examined (Figure 4). In *E. coli*, the protein accumulated to about 7% of the *total* cellular protein with pE1 (tac promoter), pE3a, and pE3b (pL promoter) and to about 40% with pE2 (T7 RNA pol promoter). In yeast, the GAPDH–ADH-2 fused promoter of pE4a and pE4b provided TS–DHFR at about 7% of the total protein.

Plasmids pE1 and pE3a also complement TS-deficient *E. coli* x2913. Transformed colonies established on minimal medium plates regardless of whether a Thy supplement was provided, although the colonies were small and grew slowly. Strain x2913 or pBR322/x2913 did not grow in Thy$^-$ medium. DHFR and TS activities for the transformants pE1 and pE3a were about half that obtained when the corresponding vectors were grown in the inducible expression systems described (Table II).

The *soluble fractions* of *E. coli* expression systems also showed bands on SDS–PAGE that corresponded to TS–DHFR, but these represented only about 1% of the total soluble protein. Inspection of *E. coli* expressing TS–DHFR by phase-contrast microscopy revealed dense inclusion bodies (Shoemaker et al., 1985) in over 90% of cells. Protein of inclusion bodies isolated from *E. coli* harboring pE2 (T7 RNA pol promoter) was 85% TS–DHFR. In contrast to the bacterial expression systems, over 90% of the expressed protein was retained in the soluble fraction of the extracts obtained from *S. cerevisiae* harboring pE4a and pE4b with the GADPH–ADH-2 promoter. SDS–PAGE of the soluble yeast extracts showed a dense band corresponding to TS–DHFR that represents about 10% of the soluble protein (Figure 5).

There was substantial catalytic activity of both DHFR and TS in *S. cerevisiae* and *E. coli* soluble extracts (Table II). Crude extracts of pE4a-transformed yeast demonstrate enzyme activities about 90-fold higher than wild-type *L. major*, 10-fold higher than antifolate-resistant *L. major* (Meek et al., 1985), and about 2000-fold higher than reported for wild-type yeast (Taylor et al., 1982). Specific activities in pE4a-transformed yeast are 5–10-fold higher than those observed in *E. coli* expressing TS–DHFR, consistent with the observed differential solubility of the bifunctional protein in yeast versus *E. coli*. Crude extracts of pE4b-transformed *S. cerevisiae* demonstrate activities that were 10% of those observed in pE4a-transformed yeast. We currently have no explanation for this. In *soluble extracts* of transformed *E. coli*, the tac promoter (pE1) gave about 17% of the activities observed in pE4a-transformed yeast,

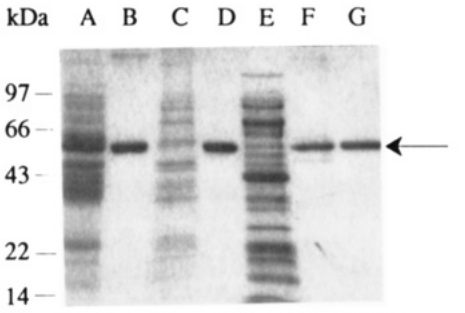

FIGURE 5: Purification of recombinant TS–DHFR expressed in *E. coli* (pE1) and *S. cerevisiae* (pE4a and pE4b), 12% SDS–PAGE stained with Coomassie-R250. In all cases, 0.5 μg of purified TS–DHFR was loaded. Arrow indicates TS–DHFR. (Lane A) pE4a-transformed *S. cerevisiae*, crude soluble extract; (lane B) pE4a-transformed *S. cerevisiae*, MTX–Sepharose-purified TS–DHFR; (lane C) pE4b-transformed *S. cerevisiae*, crude soluble extract; (lane D) pE4b-transformed *S. cerevisiae*, MTX–Sepharose-purified TS–DHFR; (lane E) pE1-transformed *E. coli*, crude extract; (lane F) pE1-transformed *E. coli*, MTX–Sepharose-purified TS–DHFR; (lane G) TS–DHFR purified from CB3717-resistant *L. major*.

the T7 RNA pol promoter gave 13%, and the pL promoter (pE3a) gave 7%. These activities are still significantly higher (6–16-fold) than that observed in wild-type *L. major* and even exceed (~about 2-fold increase) those in crude extracts of the antifolate-resistant *Leishmania* strains.

Using the specific activity values of the purified proteins, we calculate that the catalytically active DHFR represents about 2% of the total soluble protein of pE1 and pE2, 1% of pE3a and pE4b, and 13% of pE4a. However, TS activity was 40–60% that expected from the highest ratio of DHFR/TS we have observed (Meek et al., 1985). We believe this reflects decreased TS activity since TS is quite susceptible to proteolysis[2] (Garvey & Santi, 1985) and we see apparent degradation products in Western blots.

Purification and Characterization of TS–DHFR. The TS–DHFR in extracts from pE1-transformed *E. coli* and pE4(a and b)-transformed yeast were purified to apparent homogeneity by MTX–Sepharose affinity chromatography (Meek et al., 1985).[3] From a 2-L culture of pE1-transformed *E. coli* cells, 3.2 mg of purified protein was obtained in about 40% yield. From a 500-mL culture of pE4a-transformed yeast, 4.2 mg of TS–DHFR was obtained in about 20% yield; from a 500-mL culture of pE4b-transformed yeast, 1.4 mg of TS–DHFR was obtained in 40% yield (Table III). In all cases, the purified bifunctional protein exhibited a single band with M_r ~6000 by SDS–PAGE (Figure 5). The purified, recombinant bifunctional protein expressed in transformed *E. coli* or yeast exhibited DHFR specific activities that were near

Table III: Purification of *Leishmania* TS–DHFR

organism (vector)	purification step	total protein[a] (mg)	dihydrofolate reductase (unit/mg)[b]	dihydrofolate reductase total act. (units)	thymidylate synthase (unit/mg)[b]	thymidylate synthase total act. (units)	purification (*x*-fold)	yield[c] (%)
S. cerevisiae	crude extract	161	1800	289 800	181	29140		100
(pE4a)	MTX–Sepharose	4.2	15100	54 180	1045	3740	7	19
S. cerevisiae	crude extract	437	179	78 200	11	4800		100
(pE4b)	MTX–Sepharose	1.4	18250	30 100	1400	2300	120	38
E. coli[d]	crude extract	130	163	20 950	12	1540		100
(pE1)	MTX–Sepharose	0.8	8600	8 560	640	510	66	41
Leishmania[d,e]	crude extract	12.2	179	2 170	40.6	500		100
	MTX–Sepharose	0.1	14620	1 500	3620	370	82	69

[a] From 0.5-L culture. [b] Specific activities of MTX–Sepharose purified preparations are medians of at least three assays at different times; the day to day variability in TS assays of the same preparation was as high as ±20%. [c] Based on DHFR activity. [d] Values corrected from 2-L culture. [e] Data from Meek et al. (1985).

optimal, but TS activities which were 20–50% of the best preparation from *Leishmania* (Meek et al., 1985). The variability in TS activity may be accounted for by differences in N-terminal blocking (see below), exopeptidase/autolytic degradation, or folding of TS.[2]

Reverse-phase HPLC of the purified TS–DHFRs expressed in *L. major*, pE1-transformed *E. coli*, and pE4-transformed yeast demonstrated a single coeluting peak at about 49% ACN/0.1% TFA. N-Terminal sequencing of the TS–DHFR expressed in *E. coli* gave residues 2–20 as predicted from the DNA sequence (Grumont et al., 1986; Beverley et al., 1986). N-Terminal analysis of the enzyme from yeast (pE4a) gave no product, indicating that it was blocked. This was verified by showing that mixture sequencing of the CNBr digest gave all of the 13 expected N termini of the CNBr fragments. The peptide sequence for the *E. coli* recombinant traverses both the *Xba*I and *Pvu*II sites used in constructing the clones and therefore verifies the integrity of these sites and the intervening sequence. TS–DHFR purified from *L. Major* and pE4a-transformed *S. cerevisiae* showed p*I* = 6.4, while the purified preparation from *E. coli* has p*I* = 6.7. The substrate K_m values for DHFR (micromolar H_2folate, NADPH) and TS (micromolar dUMP, CH_2-H_4folate) were as follows: pE4a-yeast DHFR (1.8, 0.68), TS (0.90, 45); pE1-*E. coli* DHFR (1.3, 0.71), TS (6.0, 51); *L. major* DHFR (1.6, 0.45), TS (1.7, 80). We do not consider the differences sufficiently significant to warrant unusual interpretation.

Large-Scale Expression of TS–DHFR in Yeast. A 200-L fermenter containing YPD, 2% dextrose, 5 mg/L Trp, 5 mg/L adenine, 30 mg/L pantothenate, and 30 mg/L myoinositol was inoculated with 1 L of pE4a-transformed yeast grown in SD-leucine overnight to 0.2 OD_{600} and grown at 30 °C. After 22 h, the glucose was <8 mg/mL (Chemstrip bG; Boehringer Mannheim), and the OD_{600} was 17; in a pilot run, DHFR activity was at the onset of expression at this time. At 45 h (OD_{600} = 21), cells were harvested to yield 3.2 kg of cells, which were divided into 32 plastic bags and stored at −80 °C. The soluble extract from one of these (85 mL; 44 mg of protein/mL) showed specific activities of 627 for DHFR and 67 for TS; this is equivalent to about 135 mg of TS–DHFR or a total of 4.3 g for the 200-L fermentation. Purification of 85 mL of the extract on MTX–Sepharose gave 73 mg of homogeneous TS–DHFR (42% yield) that had specific activities of 17 344 for DHFR and 1213 for TS. This is equivalent to 2.3 g of pure TS–DHFR from the 200-L fermentation, which we will process as needed.

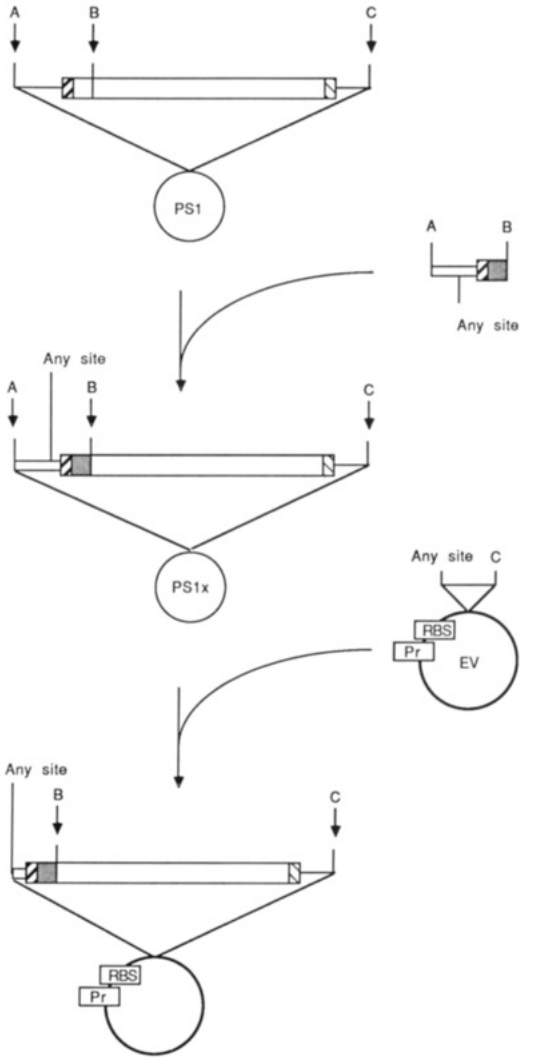

FIGURE 6: General strategy for cassette cloning of the TS–DHFR coding sequence into expression vectors (EV). The coding sequence (open bar) in a parent cloning plasmid is excised at site A, 5′ to the start codon, and site B, 3′ to the start codon. A synthetic oligonucleotide (shaded bar) containing the excised coding sequence and any unique 5′ cloning site is inserted to construct the cloning vector pS1x. The entire coding sequence is removed at the introduced unique site ("any site") and C and inserted into corresponding sites of the expression vector containing a promoter (Pr) and ribosome binding site (RBS).

DISCUSSION

Cloning Strategy. We devised a flexible cloning system that permits cassette mobilization of the entire 1560-bp coding sequence of the *L. major* TS–DHFR. The strategy (Figure 6) was to construct a parent cloning vector that would permit facile introduction of unique 5′ restriction sites anywhere near the start codon and which contained several restriction sites 3′ to the stop codon (Figure 1). This permits excision of the TS–DHFR coding sequence in toto and its precise placement

into cloning sites of numerous expression vectors (Figure 2). We cloned the entire TS-DHFR gene in a parent vector that has unique restriction sites that flank the entire coding sequence (sites A and C); next, a unique site was chosen (or constructed as a silent restriction site) downstream from and in close proximity to the start codon (site B; Figure 6). Cleavage at sites A and B, followed by insertion of a synthetic adapter, reconstructs the 5'-coding sequence and introduces any unique cloning site at or upstream of the start codon. The coding sequence can then be excised (at the introduced site and site C) and precisely placed into high-expression vectors engineered to accept the initiation codon in specific positions optimized for expression (Figure 2).

Since there are no unique restriction sites closely adjacent to the 5'-end of the coding sequence, the sequence was reconstructed from composite fragments of genomic DNA. The TS-DHFR coding sequence contains a single *Bgl*II site (Figure 1) that separates the amino-terminal DHFR and carboxy-terminal TS domains (Grumont et al., 1986; Beverley et al., 1986). The sequence corresponding to the TS domain (*Bgl*II$_3$-*Sph*I; Figure 1) was cloned into a pEMBL18 vector containing a polylinker that was modified to suit our needs. In a separate construct, we cloned the sequence containing the DHFR domain from the unique *Eco*RI site in the 5' untranslated region to the Bg*l*II$_3$ site separating the two domains; this fragment possesses a unique *Pvu*II site 13 bp 3' to the initiation codon. Replacement of the *Eco*RI-*Pvu*II fragment with an adapter containing a silent *Xba*I site (site B) at nucleotide 4 of the coding region provided plasmid pK7s, which has the flexibility needed for constructing the desired 5' cloning sites. The *Eco*RI-*Bgl*II$_3$ fragment was excised from pK7s and cloned adjacent to the TS sequence in pEMBL18m(TS), thus completing the final parent plasmid pS1.

The *Eco*RI-*Xba*I fragment (sites A and B) of plasmid pS1 was excised and replaced with synthetic adapters containing *Nco*I or *Nde*I sites at the start codon; this permitted removal of the entire TS-DHFR coding sequence and its insertion into a variety of high-expression vectors (Figure 2). In this manner, the TS-DHFR coding sequence was placed 3' to the tac (pE1), pL (pE3a and pE3b), and T7 RNA pol promoters (pE2) in *E. coli* expression vectors and 3' to the GAPDH-ADH-2 promoter in the yeast shuttle vector pBS100. In addition to the cassette approach, the TS-DHFR gene was cloned into the *Nco*I/*Sal*I sites of the yeast shuttle vector pBS100 by a more tedious, multiple-fragment ligation method (Figure 3). *Bam*HI cassettes from the yeast shuttle vectors were cloned into the yeast 2μ expression vector PC1/1 to give pE4a and pE4b.

Expression. L. major TS-DHFR was highly expressed in both *E. coli* and *S. cerevisiae* by using all vectors examined (Figure 4, Table II), although *Leishmania* DNA has a high G + C content and does not share preferred codon use with either host (Grumont et al., 1986). In *E. coli*, the levels of expressed protein were quite high, amounting to 7–40% of the total cell protein depending upon the promoter used (Figure 4). The T7 RNA pol promoter (pE2) was most impressive, showing 40% of the total cell protein expressed as TS-DHFR; however, most of the enzyme was present as insoluble inclusion bodies. In contrast, the majority of enzyme expressed in transformed yeast was found in soluble extracts and amounted to about 10% of the soluble protein. Western blot analysis indicated the presence of several minor bands, especially notable in pE2 and pE4a (Figure 4, lanes J and L), which are presumably products of TS-DHFR proteolysis (Garvey & Santi, 1985).

The soluble protein expressed in *E. coli* or yeast has catalytic activity for both TS and DHFR. In *E. coli* the active enzyme produced is at 6–16-fold higher levels than in wild-type *Leishmania* and at levels equivalent to that produced in the antifolate resistant line (Table II). From specific activity calculations, about 12 mg of TS-DHFR is present in the 250 mg of crude soluble protein obtained per liter of culture volume of pE1-transformed *E. coli*. Further, the TS-deficient host x2913 is complemented upon transformation with the expression vectors pE1 and pE3a that contain the TS-DHFR gene. The amount of active TS-DHFR produced in yeast is even more impressive. Crude extracts contain almost 100-fold more enzyme than do extracts from wild-type *Leishmania* and about 10-fold more than the antifolate-resistant protozoans (Table II). About 40 mg of TS-DHFR is present in the 320 mg of crude protein from a liter culture of yeast harboring pE4a.

Purification of recombinant TS-DHFRs was achieved by MTX-Sepharose affinity chromatography. The purified proteins had adequate DHFR specific activities but suboptimal TS activity. These differences may in part be due to degradation of the TS domain[2] and in part be due to different posttranslational modifications (see below). Up to 10 mg of homogeneous TS-DHFR has been obtained from 1 L of pE4a-transformed yeast culture and 1.5 mg from 1 L of pE1-transformed *E. coli* culture; this compares to 0.2 mg of pure enzyme obtained from a liter of MTX-resistant *L. major* promastigotes. The overall yields varied from 20 to 40% after affinity chromatography, but no attempt has yet been made to optimize the purification.

The purified TS-DHFRs from yeast, *E. coli*, and *L. major* migrated identically on SDS-PAGE and RP-HPLC. The authentic protein from *L. major* has at least one posttranslational modification; it is blocked at the N terminus (Meek et al., 1985). The TS-DHFR from yeast did not yield a PTH amino acid upon Edman degradation, and we conclude it is also blocked. In contrast, N-terminal sequencing of the enzyme expressed in *E. coli* provided the amino acid sequence from Ser-2 to Ala-20, as deduced from the DNA sequence (Grumont et al., 1986; Beverley et al., 1986). Thus, the amino terminal of TS-DHFR expressed in *E. coli* is not blocked, and the *N*-formyl-Met has been processed from the N terminus. The isoelectric points of the TS-DHFRs from recombinant yeast and *L. major* (p*I* = 6.4) versus that from recombinant *E. coli* (p*I* = 6.7) are consistent with the differences in N termini. Substrate K_m values for DHFR and TS of the enzyme expressed in yeast were essentially the same as those from *L. major*. The K_m for dUMP with the enzyme expressed in *E. coli* was somewhat higher, but we are not certain this is not due to experimental artifact.

In a 200-L fermentation of our yeast expression system, we obtained crude extract that contains over 4 g of TS-DHFR. On the basis of the yield obtained upon purification of our first batch, we calculate that we have the equivalent of over 2 g of homogeneous protein in this preparation. Considering the large amounts of TS-DHFR that can now be obtained, the accessibility of this protein has turned from low in *Leishmania* to high in heterologous expression systems.

With the availability of high expression and complementation systems for *L. major* TS-DHFR, we are now able to directly address questions regarding structure and function of this protein. In particular, mutant proteins may reveal how the domains and subunits interact and provide insight on the phenomenon of substrate channeling observed in this system (Meek et al., 1985). We also now have the ability to obtain

sufficient amounts of protein to seriously consider studies on X-ray crystallographic analysis of TS–DHFR. Such studies are timely since the structures of monofunctional counterparts of both domains are known [see Blakley (1984) and Hardy et al. (1987)], and with suitable crystals, it should not be a difficult task to solve the structure of the bifunctional protein. This structure will provide an important tool for anticipated studies on the design of specific inhibitors of protozoan TS–DHFR and could provide insight into the molecular basis of substrate channeling.

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