Amplification of protein expression in a cell free system

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ABSTRACT

Large quantities of a catalytically active protein have been produced in a cell free system. More than 10^9 copies of protein were produced from each DNA plasmid containing DNA_{fol}, the bacterial gene encoding dihydrofolate reductase (DHFR). The strategy employed, denoted gene amplification with transcription/translation (GATT), involves sequential coupling of (i) DNA amplification by the polymerase chain reaction (PCR) and (ii) *in vitro* RNA transcription by T7 RNA polymerase, followed by (iii) translation of the run-off transcripts in a rabbit reticulocyte system. The protein product had the expected size (18 kDa) and catalyzed the NADPH-dependent reduction of 7,8-dihydrofolic acid to 5,6,7,8-tetrahydrofolic acid as efficiently as authentic DHFR. Potential applications of the strategy include large scale production of enzymes containing synthetic amino acids and facilitation of the characterization of the function of genes encountered in genomic mapping studies.

INTRODUCTION

Since the initial report of the enzymatic amplification of β -globin genomic sequences (1,2), the polymerase chain reaction (PCR) has quickly been adapted for the implementation of a number of powerful biochemical strategies (3–6). These have included enhancement of the sensitivity of analysis of DNA sequence (7) and genetic phenomena (1,8,9) as well as the development of simple methods for site-directed mutagenesis (10–12), and the construction of hybrid genes (13,14). MacFerrin et al. have used PCR to facilitate the construction of expression plasmids for protein overexpression *in vivo* (15).

The elaboration of proteins *in vitro* from PCR-amplified DNA was first demonstrated by Olsnes et al. (16) for the expression of diphtheria toxin. Analogous *in vitro* studies have been reported by other workers as well (17–20), although product characterization has generally been limited to SDS-PAGE analysis of the derived proteins.

Our interest in utilizing misacylated tRNA's (21–26) in *in vitro* protein biosynthesizing systems for the preparation of proteins containing synthetic amino acids at one or more predetermined sites (27–31), has prompted us to consider strategies for the large scale production of protein *in vitro*. Because PCR-mediated DNA amplification can be effected in a medium similar to those used

for transcription and translation, we sought to sequentially couple the processes. We describe a general strategy that employs gene amplification with transcription/translation (GATT) for the cell free overexpression of proteins. This approach is illustrated for the production of dihydrofolate reductase (DHFR) starting from limited amounts of a plasmid containing the gene encoding this enzyme (DNA_{fol}) (Fig. 1). Following ($>10^6$ -fold) PCR-mediated DNA amplification, *in vitro* RNA transcription and protein translation were carried out with little or no manipulation of the intermediate DNA or RNA. Synthetic DHFR was indistinguishable from an authentic sample of the enzyme; quantification indicated that $>10^9$ copies of DHFR can be produced from each DNA plasmid employed.

EXPERIMENTAL SECTION

T7 RNA polymerase, RNasin ribonuclease inhibitor and restriction endonucleases *Bam* HI and *Pvu* II were purchased from Promega; nuclease-treated rabbit reticulocyte lysate was from Promega or Amersham. *E. coli* JM101 was obtained from Pharmacia LKB; [3 H]leucine was from ICN Radiochemicals. Nensorb-20 nucleic acid purification cartridges were from Dupont-New England Nuclear. *Taq* polymerase was purchased from Perkin-Elmer Cetus.

Plasmid pTZRKE, a derivative of pTZ19R (U.S. Biochemicals) encoding wild type *E. coli* DHFR (32) under the control of a T7 promoter, was constructed by Mr. G. Komatsoulis and generously provided by Dr. J.N. Abelson, California Institute of Technology. *E. coli* JM101 was transformed with pTZRKE and employed as the source of DNA.

DNA isolation

Isolation of plasmid DNA for PCR amplification was carried out by a lysozyme SDS-alkali procedure (33). Freshly transformed cells were grown overnight from single colonies in 3 ml of SOC media supplemented with 100 μ g/ml of ampicillin at 37°C. The culture was harvested in a 1.5-ml Eppendorf tube and resuspended in 200 μ l of 50 mM Tris-HCl, pH 8.0, containing 10 mM EDTA, 200 μ g/ml of lysozyme and 100 μ g/ml of RNase A. After incubation at 25°C for 10 min., the solution was treated with 200 μ l of 0.2 N NaOH and 1% sodium dodecyl sulfate (SDS), and mixed gently. Then 200 μ l of 2.6 M KOAc (pH 4.8) was added and the mixture was centrifuged for 15 min. (12,000 $\times$ g). The supernatant was phenol extracted and treated with 2.5 vol

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Figure 1. Schematic representation of gene amplification with transcription/translation (GATT).

of cold EtOH. The crude DNA precipitate was resuspended in 10 μ l of 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA. A typical preparation afforded 5–15 μ g of plasmid DNA after isolation on QIAGEN-tip 20 (QIAGEN Inc.). The isolated plasmid DNA was assayed by electrophoresis on 1% agarose gels, and by restriction enzyme digestion analysis.

PCR amplification of DNA

The PCR primers included a 20-nt (+) strand oligonucleotide (5'-GGCACGACAGGTTTCCCCGAC-3') complementary to a segment of pTZRKE 226–245 nucleotides upstream from DNA_{fol} and a 23-nt (–) strand oligonucleotide (5'-CTCTAGAGTCGCCGATAAGACG-3') complementary to pTZRKE 45–67 nucleotides (nt) downstream from DNA_{fol} which contains a T7 promoter followed by the DHFR open reading frame (32). These primers directed the synthesis of a duplex 791 base pairs (bp) in length. The DNA oligonucleotides were prepared on a Biosearch Model 8600 DNA synthesizer using phosphoramidite chemistry. The PCR amplification was carried out using *Taq* DNA polymerase, essentially as described (2). Plasmid pTZRKE was initially digested with restriction endonuclease *Pvu* II (to reduce non-specific product amplification), then added directly to a reaction mixture (100 μ l total volume) containing 20 mM Tris-HCl, pH 8.4 (at 25°C), 1.5 mM MgCl₂, 1 μ M DNA primers, 200 μ M dATP, dCTP, dGTP and dTTP, 50 mM KCl, 0.1% gelatin and 2 units of *Taq* DNA polymerase. The sample was overlaid with ~100 μ l of mineral oil and subjected to 30 cycles of amplification. The temperatures and incubation times were as follows: the sample was denatured at 94°C for 4 min (1 min in subsequent cycles), cooled to 50°C for 1.5 min to anneal the primers, and heated to 72°C for 2 min for primer extension. In subsequent cycles, the extension time was increased by 3 sec/cycle, and then to 9 min for the final cycle. Aliquots (5–10 μ l)

Figure 2. Amplification of DNA_{fol} by the polymerase chain reaction. A 250-ng sample of *Pvu* II-digested pTZRKE DNA (lane 2) was compared with DNA synthesized from varying amounts of the plasmid DNA in the PCR reaction. Lanes 3–7 contained the product of 44, 11, 22, 0.4 and 0.04 fmoles of DNA, respectively. PCR was carried out as described in the Experimental Section and analyzed on a 1% agarose gel. Lambda DNA treated with restriction endonuclease *Hin* dIII was used as a molecular weight marker (lane 1); the bands shown had 23130, 9416, 6557, 4361, 2322 and 2077 bp in order of decreasing size.

Figure 3. Analysis of the mRNA produced by transcription of linearized pTZRKE DNA and PCR-amplified plasmid DNA. The 597-nt mRNA produced by transcription of *Bam* HI-linearized pTZRKE DNA (250 ng and 25 ng in lanes 2 and 3, respectively) was analyzed in comparison with the mRNA produced by successive PCR amplification and transcription of 0.32 ng of plasmid DNA (lane 4). Lane 1 contained an RNA marker 679 nt in length. A control reaction involving transcription of a PCR incubation mixture carried out in the absence of plasmid DNA afforded no mRNA band (not shown).

Table 1. Quantitation of dihydrofolate reductase synthesis and activity

mRNA (μ M)	mRNA cap	DHFR synthesized/ mRNA	Specific activity ^a
0.10	–	8.8	609
	+	31	525
0.25	–	3.8	525
	+	30	633

^aSpecific activity is expressed as dihydrofolate molecules reduced/min/enzyme molecule. Authentic *E. coli* DHFR has a specific activity of 600 $\pm$ 50 (41).

were analyzed by electrophoresis on a 1% agarose gel (100 V, 10 hr, 0.5 μ g/ml of ethidium bromide in 90 mM Tris-borate, pH 8.0, containing 2 mM EDTA).

RNA transcription

RNA transcription was carried out as described (34) in an incubation mixture (20 or 100 μ l) containing 40 mM Tris-HCl, pH 7.5, 2 mM spermidine, 6 mM MgCl₂, 10 mM dithiothreitol, 10 mM NaCl, 1 unit/ μ l of RNasin, 500 μ M ATP, CTP, GTP and UTP, and 0.5 unit/ μ l of T7 RNA polymerase. An appropriate amount of *Bam* HI-linearized pTZRKE or amplified DNA_{fol} fragment was added as a template; some reactions also contained 500 μ M m⁷GpppG to effect capping of the RNA transcript (35). Transcription yields were monitored by inclusion of 50 μ Ci of

Figure 4. Analysis of dihydrofolate reductase produced from amplified and unamplified DNA_{fol}. Translation of the individual mRNA's was carried out in the presence of [³H]leucine as described in the Experimental Section; the [³H]-labeled proteins were analyzed by SDS-PAGE and fluorography. Lane 1 contains [³H]DHFR produced from mRNA derived from unamplified DNA_{fol}; lane 3 contains [³H]DHFR derived from amplified DNA_{fol}. A control experiment that employed the product of a PCR amplification carried out in the absence of plasmid DNA produced no detectable protein band (lane 2). Proteins translated from BMV RNA were employed as molecular weight markers (kDa).

A time course

0 0.5 1.5 3 6 9 hr

B effect of RNA concentration

Figure 5. Production of DHFR as a function of time and RNA concentration. Time dependent production of DHFR (upper panel) was carried out using 0.25 μM RNA from PCR-amplified DNA_{fol}. Production of DHFR as a function of RNA concentration (lower panel) was measured after a 90-min incubation at 30°C.

[α-³²P] CTP in the reaction mixtures. Aliquots were analyzed by polyacrylamide gel electrophoresis (PAGE) (5% denaturing gel, 90 mM Tris-borate, pH 8.0, containing 8 M urea and 2 mM EDTA); the products were detected by autoradiography. The yield of RNA transcript was typically 10 μg of RNA/μg template.

Figure 6. Binding of synthesized DHFR to a methotrexate affinity column. Five μl of a reaction mixture containing [³⁵S]methionine, rabbit reticulocyte lysate and the mRNA for DHFR treated with methotrexate agarose (51). Lane 1, crude reaction mixture. Lane 2, supernatant remaining after methotrexate-agarose resin was removed from the reaction mixture. Lane 3, protein eluted from methotrexate agarose by heating the resin in SDS-gel loading buffer. The samples were analyzed on a 15% SDS-PAGE gel with a 5% stacking gel (run initially at 50V and then at 100V) in 25 mM Tris-glycine buffer, pH 8.3, containing 0.1% SDS. The gel was visualized by autoradiography. The position at which protein molecular weight markers migrated is indicated (kDa).

DHFR translation

DHFR translation was carried out in a reaction mixture (30–50 μl total volume) that typically contained 58% leucine-depleted, nuclease-treated rabbit reticulocyte lysate, 40 μM amino acids except leucine, 12–18 μM [³H]leucine (110 Ci/mmol), 20 μM unfractionated *E. coli* tRNA's, 1 unit/μl of RNasin ribonuclease inhibitor and 0.1–3.3 μM RNA transcript (or 2–5 μl of unpurified transcription reaction mixture). The reactions were incubated at 30°C and (5–10 μl) aliquots were removed for analysis of [³H]-labeled proteins by SDS-PAGE (16.5% acrylamide, 0.1% SDS) (36) and fluorography (37).

Translation of the RNA transcript was also carried out in the presence of [³⁵S] methionine in order to enhance the detection of other proteins elaborated by rabbit reticulocyte lysate. The reaction mixture (50 μl total volume) included 40 μl of lysate and ~5 μl of unpurified transcript in addition to [³⁵S]methionine at a final concentration of 0.8 mCi/ml. Following incubation at 30°C for 1 hr, a 10-μl aliquot was treated with methotrexate-agarose for 1 hr at 25°C. The recovered resin was washed four times with water, then heated in the presence of SDS-gel loading buffer (50 mM Tris-HCl, pH 6.8, containing 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) at 100°C for 5 min for SDS-PAGE analysis. The supernatant, and an aliquot of the reaction mixture not treated with methotrexate-agarose, were denatured in the same fashion prior to SDS-PAGE analysis.

RESULTS AND DISCUSSION

DHFR plays an important role in the biosynthesis of DNA, some amino acids and numerous cellular metabolites (38–40). Characterization of the enzyme has been facilitated by its small size (~18 kDa), water solubility, lack of post-translational modifications, and propensity to fold into its catalytically active form. DHFR can also be isolated readily and assayed for catalytic function with facility (41–43). In the aggregate, these properties made DHFR an excellent candidate for overexpression in a cell free system.

Plasmid pTZRKE encoding the *Escherichia coli* DHFR gene (*fol*) (32), prepared from approximately 10⁷ cells by a

microscale procedure, gave ample DNA ($\sim 5\text{--}15\ \mu\text{g}$) for the studies described here. Specific amplification of the *fol* gene was achieved by the use of primers complementary to oligonucleotide sequences that flank the desired gene. After 30 cycles of PCR (2), the amplified DNA was analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide. As shown in Fig. 2, a band corresponding to the amplified DNA_{*fol*} (791 bp) was readily apparent and increased in rough proportion to the amount of plasmid DNA initially present. Approximately $10^5\text{--}10^6$ copies of DNA_{*fol*} were obtained for each DNA plasmid employed in these incubations; PCR amplification was maximally (70–85%) efficient when 1–10 ng (0.4–4.0 fmoles) of template was employed. The amplified DNA was used directly for transcription of RNA.

Linearized plasmid pTZRKE DNA and amplified DNA_{*fol*} were compared for their abilities to direct the synthesis of the expected 597-nt RNA transcript. As shown in Fig. 3, both templates produced RNA products of the same length (lanes 2–4). Quantification of the RNA transcript indicated that $\sim 1.2 \times 10^6$ copies of RNA had been produced from each of the plasmid DNA's originally utilized for amplification. In other experiments (not shown), the mRNA/plasmid DNA ratio was about 70-fold better (ie, $\sim 10^8$ copies of RNA for each plasmid DNA). The RNA transcript prepared from the PCR-amplified DNA was used directly for translation of DHFR.

The RNA's produced by *in vitro* transcription were compared for their abilities to direct the synthesis of DHFR in a cell free protein biosynthesizing system. We employed a nuclease-treated rabbit reticulocyte lysate system for its sensitivity to added mRNA, favorable rate of protein synthesis, absence of extraneous reactions, and stability of mRNA and elaborated protein (44). The results are illustrated in Fig. 4, for an experiment that employed 0.25 μM mRNA derived either from linearized pTZRKE DNA, or from the PCR-amplified plasmid, in the presence of [^3H]leucine. Fluorography of the SDS-PAGE revealed the presence of a single band having the mobility expected for DHFR. That both incubations afforded the same amount of product was determined both from the intensities of the bands in the fluorograms, and by determination of radioactivity in the appropriate gel slices. The abilities of the derived proteins to catalyze the NADPH-dependent reduction of 7,8-dihydrofolic acid to 5,6,7,8-tetrahydrofolic acid were also assayed (39); the putative DHFR's catalyzed this transformation essentially as well as DHFR isolated from *E. coli* (41–43), when the amount of synthesized DHFR was calculated from its [^3H]leucine content, assuming 11 leucines per DHFR (32). Quantification of some of the data is presented in Table 1.

Although PCR-amplified DNA has been reported to contain inhibitors that can affect translation of the derived RNA (20,45), and the ultimate polypeptide products have been shown to include incomplete or degraded proteins, no problems of this type were noted in the present study. In contrast, the crude DNA preparations employed here could be amplified readily by PCR, and underwent *in vitro* transcription in the presence of T7 RNA polymerase. The resulting RNA transcripts produced DHFR preparations of comparable yield and quality whether or not they were purified prior to *in vitro* translation.

Because the initial translation experiments resulted in the synthesis of only 0.4–0.5 DHFR/RNA transcript, we attempted to improve translational efficiency. Investigation of the RNA concentration dependence of DHFR synthesis (Fig. 5, lower panel), indicated optimal protein production in the presence of

0.5 μM mRNA, an effect noted consistently in replicate experiments. Although polyamines and Mg^{2+} have been reported to improve both the level and fidelity of cell free protein synthesis (44,46) we observed no effect of added spermidine ($\leq 1\text{mM}$) or Mg^{2+} ($\sim 0.8\text{mM}$), although higher concentrations of either decreased the extent of DHFR synthesis. Under optimal conditions, the production of DHFR proceeded steadily over a period of 90 minutes, affording a product that was stable in the incubation mixture for at least 9 hours (Fig. 5, upper panel).

The mRNA cap structure m⁷G(5')N has been reported to enhance the extent of protein biosynthesis in eukaryotic systems (17,47–50); this structure facilitates ribosome binding during the initiation phase of translation, and stabilizes the mRNA against 5'-exonucleases. When amplified DNA_{*fol*} was transcribed in the presence of m⁷GpppG (35), a capped mRNA was obtained. Subsequent translation of this capped mRNA resulted in protein synthesis several-fold greater than that obtained using unmodified mRNA (Table 1). Optimal translation of a portion of the capped mRNA at 0.025–0.10 μM mRNA (cf Fig. 5 for the uncapped mRNA) yielded up to 31 DHFR molecules per mRNA, equivalent to $\sim 3 \times 10^9$ copies of catalytically competent DHFR per original *fol* gene copy isolated from *E. coli* cells.

To further characterize the synthesized DHFR, translation was carried out in the presence of [^{35}S]methionine, which enhanced the visualization of proteins elaborated in parallel with DHFR. As shown in Fig. 6, admixture of a quantity of methotrexate-agarose to the reaction mixture resulted in the selective binding of a single protein, subsequently shown to co-migrate with authentic DHFR after removal from the methotrexate affinity resin (lane 3). The binding of authentic DHFR to this affinity resin (51) provides further evidence that the DHFR synthesized in this study is functional.

It is anticipated that the GATT procedure can be used for the elaboration of many gene products *in vitro*, especially where the proteins of interest lack post-translational modifications, and fold into their native forms with reasonable facility. The GATT procedure should be applicable to a wide variety of molecular cloning/mutagenesis strategies. For example, had the pTZRKE *fol* gene not contained the T7 promoter, it would have been possible to have designed the PCR primers so that it was added to the 5' end of the coding sequence as a result of the PCR reaction. As another example, deletion mutants could easily be constructed by targeting a portion of the gene, rather than the full length gene, and by adding the T7 promoter to that portion along with an appropriate ribosome binding site. Other possible enhancements of the present technology might include the use of a coupled transcription-translation system (27) or the continuous flow system described by Spirin et al. (52) for enhancing protein synthesis *in vitro*. In addition to supporting a strategy for the elaboration of proteins containing synthetic amino acids at defined sites (21–29), the availability of an efficient method for protein elaboration *in vitro* may facilitate the production of certain proteins (eg. cytotoxic species, proteins that form inclusion bodies) which cannot readily be overexpressed in intact cells.

It may be noted that the extent of amplification realized in these experiments would in principle allow the biochemical expression/characterization of a single gene product from a few μg of total prokaryotic genomic DNA. Such technology may also prove useful in facilitating the characterization of gene products (53) from very large fragments of genomic DNA cloned in artificial chromosome vectors.

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